drolysis of Ap_3A, Ap_5A, Ap_6A, Ap_4, and the corresponding guanine nucleotides, biphasic kinetics for Ap_4A, and inhibition by Mg^{2+} (Barnes & Culver, 1982) are specific characteristics of the *Physarum* enzyme.

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Molecular Aspects of Bovine Erythrocyte Bis[(heme *b*)copper] Protein†

Ulrich Weser,* Alfred Gärtner, and Karl-Heinz Sellinger

ABSTRACT: An erythrocyte Cu_2(heme $b)_2$ protein of M_r 400 000 was successfully isolated. Incubating the protein in sodium dodecyl sulfate prior to polyacrylamide gel electrophoresis caused the splitting into M_r 70 000, 120 000, and 200 000 units. The copper was fully electron paramagnetic resonance detectable of the type II ($g_\perp = 2.0309$, $g_\parallel = 2.2122$, $A_\parallel = 175$ G). The high-spin ($d^5_{5/2}$) iron(III) showed a g value of 6.05. No magnetic interaction between copper and heme iron was detected. In a comparison of more than ten different enzymatic oxidase activities, not a single one could be assigned to the Cu_2(heme $b)_2$ protein. Azide, CO, cyanide, fluoride, and imidazole were bound to the heme iron. The binding of imidazolate suggests the accessibility of the sixth coordination site of the heme *b* group to fairly large ligands. Removal of the copper by ethylenediaminetetraacetic acid or cyanide resulted in an irreversible precipitation of the protein. This supports the structural contribution of the copper.

The copper concentration in both serum and red blood cells is near 10 μM (Underwood, 1971). The origin of the serum copper is fairly well understood; it is almost entirely bound in ceruloplasmin (Carrico & Deutsch, 1969a; Malkin & Malmström, 1970; Frieden, 1980). Specific copper binding sites have been characterized in this protein (Malkin & Malmström, 1970; Fee, 1975). All three copper chromophores as defined by Malkin & Malmström (1970) are detectable.

At present, only 38% of the copper content in red blood cells has been assigned to a copper and zinc containing protein called erythrocuprein (Mann & Keilin, 1938; Carrico & Deutsch, 1969b, 1970; Weser, 1973). The latter protein has had many different names and is presently called superoxide dismutase (McCord & Fridovich, 1969). The occurrence of another erythrocyte copper protein called "pink copper protein" has been reported by Reed et al. (1970). Its existence could not be confirmed (H. Deutsch et al., unpublished results; G. Rotilio et al., unpublished results; U. Weser et al., unpublished results).

In a preliminary study a Cu_2(heme $b)_2$ protein of M_r 400 000 was reported (Sellinger & Weser, 1981). While the functional side of erythrocuprein or superoxide dismutase copper is debated, at least, the biochemical role of the copper heme protein is completely unknown. It was of interest to characterize this copper heme protein in more detail. How many subunits

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0006-2960/82/0421-6133$01.25/0

contribute to the overall structure of the protein? The two copper atoms, are they extraneously bound and/or do they support the overall protein structure? Ligand binding studies were performed to shed some light on the sixth coordination sphere of the heme moiety.

Experimental Procedures

All reagents were obtained from the same sources as reported elsewhere (Sellinger & Weser, 1981). Polyacrylic columns of different sizes were employed to minimize metal contamination. Deionizied water was additionally distilled over quartz and had a conductivity of less than 0.5 μS. All buffer solutions were treated with Chelex-100 (Bio-Rad, München, German Federal Republic). The copper concentration in the buffer was below 1 nM. The isolation of Cu_2(heme b)$_2$ protein from bovine erythrocytes was performed following the procedure of Sellinger & Weser (1981) with minor modifications on DEAE-Sephacel[1] instead of DEAE-cellulose.

Heme. A solution of the desalted Cu_2(heme b)$_2$ protein was lyophilized and extracted 3 times with 5 mL of acetone (−20 °C) containing 50 μL of 2.4 M HCl. The combined extracts were evaporated and suspended in 1 mL of pyridine (Rieske, 1967). After the addition of 1 mL of 0.2 M KOH, difference spectra of the oxidized (ferricyanide) and reduced (dithionite) hemes allowed the assignment of the positions of α, β, and Soret bands.

Enzymes. Spectrophotometric assays for the following enzymes were carried out: ascorbate oxidase (Baker & Bruemmer, 1968), catalase (Bergmeyer, 1955), cytochrome *c* oxidase (Smith, 1955), cytochrome *c* peroxidase (Smith, 1955), diamine oxidase (Holmstedt et al., 1961), dopamine β-hydroxylase (Wallace et al., 1973), galactose oxidase (Avigad et al., 1961), indoleamine 2,3-dioxygenase (Hirata et al., 1974), methemoglobin reductase (Kuma, 1981), monoamine oxidase (Tabor et al., 1954), the monooxygenase reaction of cytochrome P-450 (Schenkman et al., 1967), peroxidase (*Worthington Enzyme Manual*, 1972), superoxide dismutase (Weser et al., 1980), tyrosinase (Duckworth & Coleman, 1970), and uricase (Mahler et al., 1955).

Polyacrylamide Electrophoresis. Analytical polyacrylamide gel electrophoresis was carried out according to Sokolowski & Weser (1975). For the determination of the relative molecular mass, the gels were calibrated with catalase (M_r 4 × 62000), ceruloplasmin (M_r 135000), ferritin (M_r 450000), and xanthine oxidase (M_r 275000).

Spectrometry. Ultraviolet and visible absorption was recorded on a Beckman 25 spectrometer. Circular dichroism was measured employing a Jasco J-20 A recording spectropolarimeter. Electron paramagnetic resonance (EPR) was detected at 77 K on a Varian E-109 spectrometer.

Results

Molecular Properties. During polyacrylamide gel electrophoresis using gels ranging from 5–10% w/v the protein migrated as one single homogeneous band. Relative molecular mass determinations from gel filtration and sodium dodecyl sulfate gel electrophoresis experiments indicated a M_r of 400000 ± 10%. Upon incubation of the protein for 1 h at 45 °C in the presence of sodium dodecyl sulfate, urea, and mercaptoethanol, a splitting into four different bands was seen. Prolonged incubation at temperatures up to 100 °C and in the absence of mercaptoethanol did not change the electrophoresis pattern (Figure 1). The major and fastest migrating component has a M_r 70000. Three more bands had relative molecular weights of 120000, 200000, and 400000, respectively. The band closest to the origin was considered to be the native Cu_2(heme b)$_2$ protein. It might be possible that two nonidentical subunits at 70000 and 120000 daltons form a tetrameric molecule. The oligomerization of 70000 units with the concomitant loss of some of the metals has also to be considered. Furthermore, an unspecific splitting following the sodium dodecyl sulfate incubation cannot be rejected at the moment. Unfortunately, the metal concentration in these bands was below 10^{-10} M. A decision where the metals are located could not be made as this concentration range is below the detection limit of the employed atomic absorption spectrometer. The amino acid composition was the same as earlier described (Sellinger & Weser, 1981). In contrast to ceruloplasmin (Frieden, 1980), no significant amount of carbohydrate was detected.

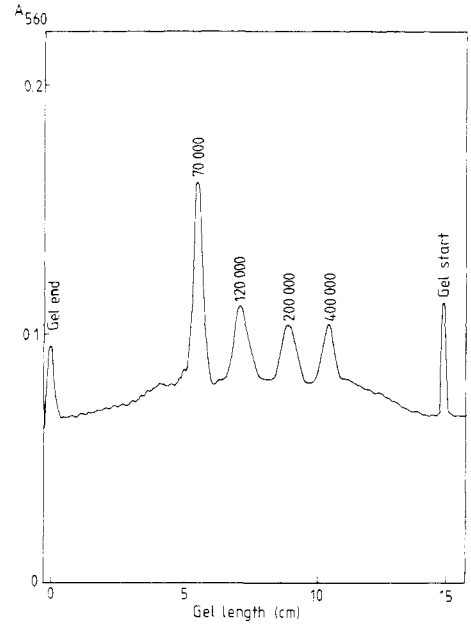

FIGURE 1: Electrophoretogram of Cu_2(heme b)$_2$ protein in 5% acrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. 50 μg of Cu_2(heme b)$_2$ protein was incubated in 4 M urea, 1% sodium dodecyl sulfate, and 1% mercaptoethanol for 1 h at 45 °C. Electrophoresis was performed at 100 V and 2 mA per gel stick for 3 h at 22 °C. Staining was with Coomassie brilliant blue. The scanning speed was 0.2 mm/s; the chart speed was 0.2 mm/s; the slit openings were 0.2 mm perpendicular and 3 mm parallel to the gel. Scanning was carried out at 560 nm. The numbers over the protein components are their molecular weights.

Analysis of the iron chromophore resulted in the assignment of all iron in a heme moiety of the *b* type. The difference spectra of the oxidized and reduced heme showed an α band at 556 nm. Unlike with catalase and horse radish peroxidase the intensity of the β band at 524 nm was close to that of the α band (Figure 2). The Soret peak was monitored at 418 nm. Absorption spectrometry proved unsuitable to characterize the copper binding site as strong contributions originating from the heme *b* chromophores overlapped possible Cu-absorption bands.

In the overall EPR spectrum the strong contribution of the heme iron high-spin signal appears at $g = 6.05$. In contrast to the EPR properties of both catalase and ferri horse radish peroxidase, no rhombic splitting in this region was detectable (Blumberg et al., 1968; Morita & Mason, 1965). The signal at $g = 4.3$ is attributed to non-heme rhombic iron (Figure 3). Quantification studies revealed that all copper was EPR de-

[1] Abbreviations: DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

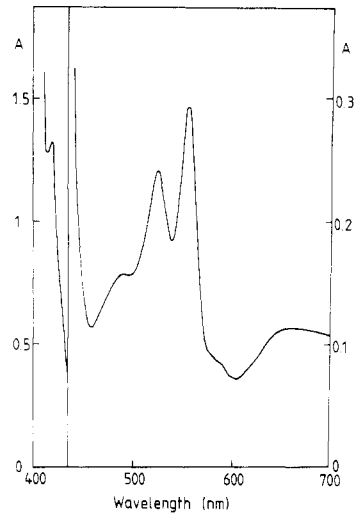

FIGURE 2: Difference spectroscopy of Cu_2(heme b)$_2$ protein chromophore in pyridine/KOH. 10 mg of protein was treated 3 times with 5 mL of cold acetone/0.05 mL of 2.4 N HCl to extract the iron chromophores. The acetone extracts were evaporated and dissolved in 1 mL of pyridine. This solution was added to an equal volume of 0.2 N KOH. One portion was reduced with excessive solid sodium dithionite. The difference spectrum between the same aliquots of the reduced and oxidized heme was run, with 10-mm cells at 23 °C.

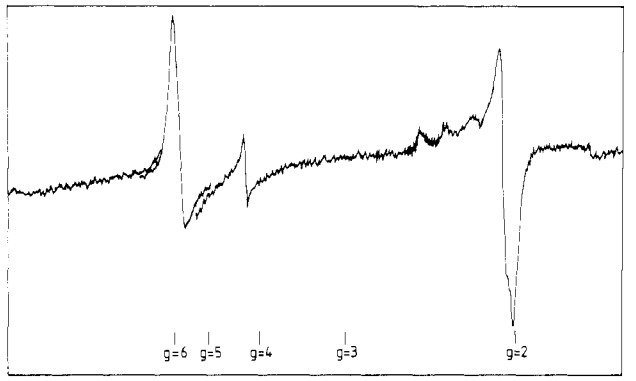

FIGURE 3: EPR properties of Cu_2(heme b)$_2$ protein. 30 mg of protein was dissolved in 1 mL of 50 mM potassium phosphate, pH 7.0. The spectrum was run on a Varian E-109 spectrometer with quartz cuvettes. The modulation amplitude was 12.5 G; the modulation frequency was 100 kHz; the microwave power was 20 mW; the microwave frequency was 9.23 GHz; the temperature was 77 K. The signal in the $g = 6$ region is due to high-spin heme iron, while the $g = 4.3$ signal arises from non-heme rhombic iron. The iron signal (g value approximately 6.0) had to be recorded with a 1.6-fold diminished gain. Quantification of the Cu EPR signal was carried out with CuEDTA as a standard.

tectable and was assigned to the type II having g values of $g_\perp = 2.0309$ and $g_\parallel = 2.2122$, and $A_\parallel$ was 175 G. Unlike with cytochrome c oxidase no magnetic coupling between copper and heme iron was seen.

Functional Aspects. The search for a biochemical function of Cu_2(heme b)$_2$ protein was unsuccessful. No measurable enzymic activity for ascorbate oxidase, cytochrome c oxidase, cytochrome c perioxidase, cytochrome P-450, diamine oxidase, dopamine β-hydroxylase, galactose oxidase, indoleamine 2,3-dioxygenase, methemoglobin reductase, monoamine oxidase, peroxidase, superoxide dismutase, tyrosinase, and uricase was found. Catalase was the only detectable activity and was only 0.1% per heme.

Ligand binding studies were carried out in attempts to shed some light on the metal binding sites. The electronic absorption recorded at 200–750 nm always resulted in a multibanded absorption in the pH region between 5.5 and 11 (Sellinger & Weser, 1981). According to Peisach et al. (1968)

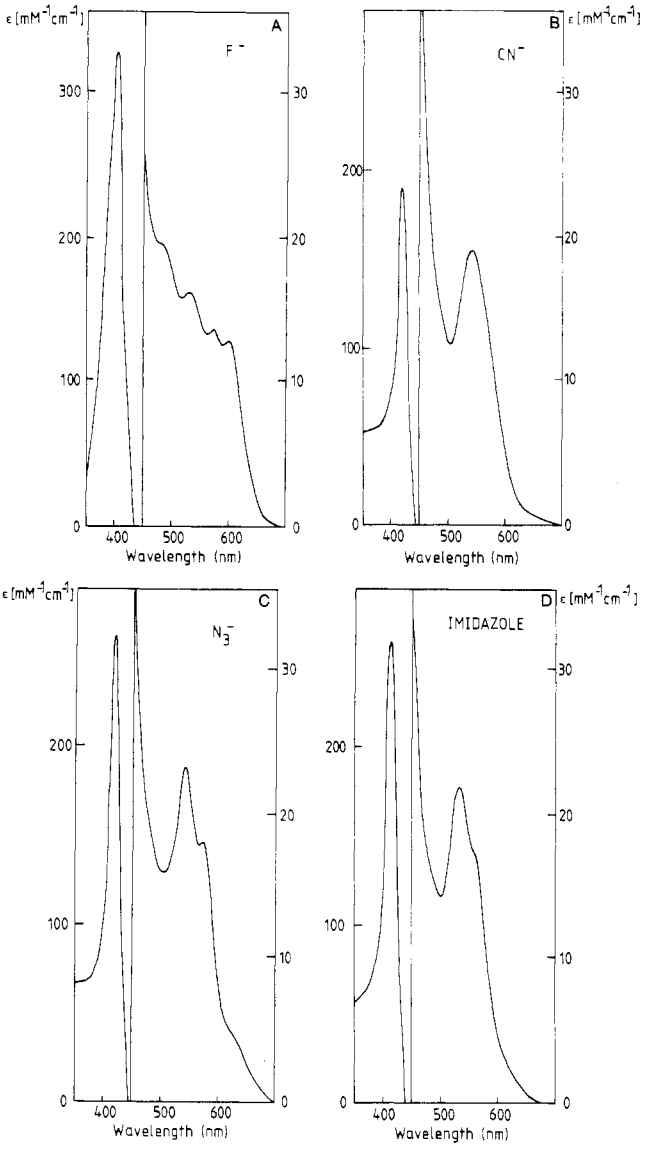

FIGURE 4: Absorption of Cu_2(heme b)$_2$ protein in the presence of different heme ligands: (A) KF, (B) KCN, (C) NaN_3, and (D) imidazole. 2 mg of the protein dissolved in 1 mL of 100 mM Tris-HCl (pH 7.0) was titrated in 10-μL steps with 1 M solutions of the respective ligand. This minimized erroneous absorption readings attributable to dilution effects. Throughout, no further spectroscopic change was detectable at a final overall concentration of 1 mM.

and Blumberg et al. (1968) the heme b iron can be assigned to the high-spin state ($d^5_{5/2}$). Upon reaction with dithionite the bands at 495, 535, and 570 nm disappeared. The absorption at 403 nm was shifted to 412 nm, and the absorption coefficient was reduced. A diminished absorption was also seen at 625 nm. The electronic structure of the ferroheme is suggested to be low spin (d^6_0).

The addition of ferricyanide resulted in the spectrum of the Cu_2(ferriheme b) protein. In the presence of fluoride the absorption profile was similar to that of the native Cu_2(heme b)$_2$ protein, suggesting high-spin ($d^5_{5/2}$) ferriheme. Furthermore, a significant sharpening of the $g = 6.05$ signal was seen. The bands at 570, 535, and 495 nm remained essentially unchanged. A blue shift was noted at 600 nm, and the absorption at 403 nm increased markedly (Figure 4A).

The addition of cyanide, azide, and imidazole resulted in absorption spectra characteristic for the ferri low-spin type (Figure 4B–D). No detectable changes were seen in the 200–300-nm region. The absorption at 418 nm was more

Table I: Comparison of Electronic Absorption Properties of Different Heme *b* Proteins and Their Derivatives [a]

heme protein	isolated protein	added ligand: ferri form: fluoride	ferri form: cyanide	ferri form: azide	ferri form: imidazole	ferro form: dithionite	ferro form: dithionite and CO
erythrocyte Cu_2(heme *b*)$_2$ protein [b]	625 (6)	600 (12)	545 (19)	625 [i] (4)	572 [i] (19)	625 [i] (4)	625 (4)
	570 (12)	570 (13)	418 (210)	572 (19)	545 (22)	550 (22)	565 (21)
	535 (16)	535 (17)		540 (22)	418 (260)	418 (170)	535 (22)
	495 (17)	495 [i] (21)		418 (270)			418 (170)
	403 (220)	403 (350)					
bovine erythrocyte catalase [b,c,d]	625 (8)	600 (14)	584 (34)	621 (30)	*h*	*h*	*h*
	540 (10)	526 (13)	550 (45)	540 (25)			
	500 (12)	485 (17)	423 (115)	502 (33)			
	403 (115)	405 (120)		418 (140)			
horseradish peroxidase [b,e,f,g]	641 (3)	612 (7)	539 (10)	635 (2)	*h*	588 [i] (7)	572 (11)
	500 (10)	488 (8)	418 (94)	534 (8)		557 (11)	542 (12)
	403 (100)	404 (130)		416 (115)		418 (77)	422 (138)

[a] The numbers represent the absorption maxima in nanometers. The values given in parentheses are millimolar absorption coefficients calculated per heme moiety. The protein concentration was 2 mg/mL in 10 mM Tris-HCl (pH 6.9). In the presence of the ligands a higher buffer concentration (100 mM Tris-HCl, pH 7.0) was used. Spectra were recorded at 23 °C in 10-mm cells. For further details, refer to the legends of Figures 4, 5, and 6. [b] This study. [c] Torii & Ogura (1968). [d] Schonbaum & Chance (1976). [e] Keilin & Hartree (1951). [f] Blumberg et al. (1968). [g] Peisach et al. (1968). [h] No reaction. [i] Shoulder.

pronounced than that of the reduced protein. Unlike cyanide, imidazolate and azide (Figure 4C) did not fully level off the 572-nm band. In contrast to catalase, azide was able to form the low-spin ferri spectrum already at room temperature (Torii & Ogura, 1968). It was of considerable interest to see the reactivity of imidazole (Figure 4D). This suggests the accessibility of the sixth coordination site of the heme *b* moiety to ligands of fairly large size.

Only reduced Cu_2(heme *b*)$_2$ protein reacts readily with CO (Figure 5). While the Soret band and the shoulder at 625 nm remained unchanged, the band at 550 nm of the reduced protein is split into two bands located at 565 and 535 nm. Regardless of whatever ligand was employed, no detectable changes were observed in the chiroptical properties. A negative Cotton band at 221 nm (θ = −880 000 grad·cm^2·dmol^{-1}) was recorded that could be attributed to α-helical regions of the polypeptide chain. No measurable chiroptical properties could be assigned to the metal chromophores.

Azide and imidazolate affected the heme *b* iron. The copper signal remained unchanged at $g_\perp$ = 2.0309. Fluoride caused a sharpening and a more intensive signal of the heme *b* iron in the g = 6 region while no detectable changes of the copper EPR signal were seen.

Attempts were made to extract the copper from the Cu_2(heme *b*)$_2$ protein. After 24-h dialysis against either 100 mM KCN at pH 10.5 or 100 mM EDTA at pH 7.4, 70% of the total copper was removed. The presence of the chelators was necessary as control dialyses did not extract the metal. Removal of copper led to irreversible denaturation. Furthermore, added $CuSO_4$ or dialysis against 0.1 mM $CuSO_4$ or dialysis against 0.1 mM $CuSO_4$ enhanced the denaturation. It may be concluded that copper is not extraneously bound but is of considerable structural and/or functional importance.

Discussion

The amount of unspecifically bound copper cannot be metabolically controlled. In addition, the distinct biochemical reactivity of copper including the copper-dependent formation of excited oxygen species (Michelson et al., 1977; Bannister & Hill, 1980; Weser et al., 1980; Weser & Schubotz, 1981) requires specific binding sites for all blood copper proteins. The copper chromophores in ceruloplasmin are fairly well understood. The same applies to the erythrocyte cuprein or Cu_2Zn_2 superoxide dismutase. In the presently described

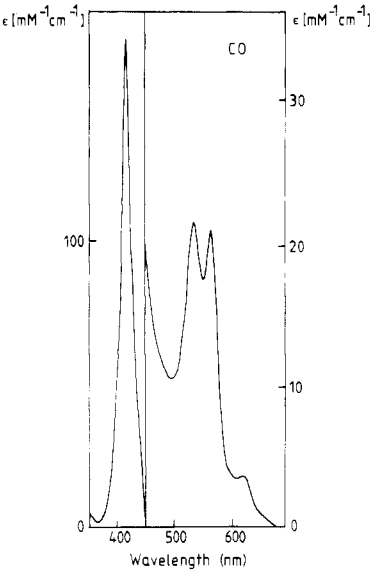

FIGURE 5: Absorption of CO-Cu_2(heme *b*)$_2$ protein. 2 mg of protein was reduced by the addition of excessive solid sodium dithionite and gassed for 1 hour with CO. The spectrum was recorded in 10-mm cells at 23 °C.

Cu_2(heme *b*)$_2$ protein there is another metalloprotein with a characteristic copper chromophore. The total amount of erythrocyte copper found in Cu_2(heme *b*)$_2$ protein has not been fully detected. If one takes into account the many different chromatographic steps leading to a final yield of 10% of the overall copper present in the hemolysate, a rough estimate would allow 20–50% to be found in this new copper protein.

Earlier attempts to isolate copper proteins other than Cu_2Zn_2 superoxide dismutase failed. This was attributed to a heme iron:copper ratio of approximately 1700, the large molecular size, and the above-mentioned lability of the Cu_2(heme *b*)$_2$ protein. As in the earlier method of Stansell & Deutsch (1965) for isolating catalase, the present isolation of the copper heme protein was successful with strictly aqueous chromatographic procedures. The probable reason why this protein was not earlier detected was attributed to the important fact that the original DEAE-cellulose step was replaced by DEAE-Sephacel. The Cu_2(heme *b*)$_2$ protein does not survive repeated DEAE-cellulose chromatography. Furthermore, the use of hydroxylapatite was both mild and efficient in separating contami-

nants. The chemical and physicochemical properties of myoglobin, oxymyoglobin, hemoglobin, oxyhemoglobin, and methemoglobin are clearly distinguishable from those of the Cu_2(heme b)$_2$ protein (Mahler & Cordes, 1971). In a comparison of the known data of catalase, a vague relationship could be seen. Very early studies showed that blood catalase was contaminated with copper (Agner, 1938). The relative molecular mass of the present monodisperse Cu_2(heme b)$_2$ protein is almost twice that of catalase. Unlike catalase the protein precipitates irreversibly in aqueous 50% ethanol or after removal of copper. The electronic absorption properties are distinctively different. In Table I the absorption bands and the millimolar ϵ values of the heme moieties of catalase and horseradish peroxidase, a heme b protein of different biological origin, are summarized. The values for catalase were obtained from freshly prepared blood catalase and agree with those described by Torii & Ogura (1968) and Schonbaum & Chance (1976). The absorption coefficients of the Cu_2(heme b)$_2$ protein are approximately 2 times higher in the 50–625-nm region. A new band appears at 570 nm that is absent in the spectrum of catalase. Catalase does not react either with imidazole, dithionite, or CO.

There are further differences in the line shape of the high-spin iron EPR signal in the $g = 6$ region. Stepwise redox titrations with dithionite revealed that cupric copper is initially reduced, leaving the $g = 6$ signal unchanged. Unfortunately, no enzymic activities were noted. Whether or not there is a missing cofactor needs to be elucidated. Both the heme b component and the copper encourage the search for a catalytic activity.

Acknowledgments

Thanks go to Dr. W. Stock, W. Voetsch, H. Heinemann, and H. Schneider for expert technical help.

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