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Studies on the bacterial hemoglobin from *Vitreoscilla*

Redox properties and spectroscopic characterization of the different forms of the hemoprotein

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Summary. *Vitreoscilla* contained a homodimeric bacterial hemoglobin (VtHb). The purification of this protein yielded VtmetHb which exhibited electronic and electron paramagnetic resonance (EPR) spectra, showing that it existed predominantly in a high-spin ferric form, both axial and rhombic components being present. The preparations also contained variable amounts of low-spin components. There was no evidence that these high-spin and low-spin forms were in equilibrium. The former were reducible by NADH catalyzed by the NADH-metVtHb reductase, and the latter were not. High ionic strength and high pH led to the formation of low-spin metVtHb; both treatments were reversible. Cyanide and imidazole liganded to VtHb resulted in the conversion of high-spin to low-spin ferric heme centers, each with characteristic electronic and EPR spectra. Some preparations of VtHb exhibited EPR signals consistent with a sulfur ligand bound to the ferric site. When VtHb was treated with NADH plus the reductase in the presence of oxygen, the intensity of the high-spin EPR signals decreased significantly. No reduction occurred in the absence of oxygen, suggesting a possible role for the superoxide anion. Dithionite treatment of VtHb resulted in a slow reduction, but the main product of the reaction of dithionite-reduced VtHb with oxygen was VtmetHb, not $VtHbO_2$. EPR spectra of whole cells of *Vitreoscilla* exhibited a variety of intense signals at low and high magnetic field, the *g*-values being consistent with the presence of high-spin ferric heme proteins, in addition to an iron-containing superoxide dismutase (FeSOD) and iron-sulfur proteins. EPR spectra of the cytosol fraction of *Vitreoscilla* showed the expected resonances for VtmetHb and FeSOD.

Key words: Bacterial hemoglobin – Oxygenated hemoglobin – High- and low-spin hemoglobin – NADH-dependent hemoglobin reductase – Iron superoxide dismutase

Introduction

Vitreoscilla hemoglobin (VtHb) was formerly called soluble cytochrome *o* until it was positively identified as the first known bacterial hemoglobin by sequence similarity with leghemoglobin (Wakabayashi et al. 1986). It is a dimer with a molecular mass of 32783 Da that contains two identical subunits and two hemes per molecule (Tyree and Webster 1978a). The ferrous form binds both CO and O_2 to form compounds with optical spectra that are similar to those of the corresponding compounds of myoglobin and hemoglobin (Liu and Webster 1974). Rapid kinetic studies of the binding of CO and O_2 to the protein suggest that it has a relatively open heme pocket which may explain why the oxygenated form ($VtHbO_2$) is more autoxidizable than most oxyhemoglobins (Orii and Webster 1986). The binding of CO to the heme centers in the reduced protein is strongly cooperative, but the ferric heme iron centers in the oxidized protein (VtmetHb) bind cyanide as two identical, independent binding sites (Tyree and Webster 1978a).The protein is normally isolated and purified in the oxidized form. A flavoprotein, NADH–metVtHb reductase (previously called NADH–cytochrome *o* reductase), was also purified from the cytosol of *Vitreoscilla*; it catalyzes the reduction by NADH of partially purified metVtHb and a number of electron acceptors such as ferricyanide, cytochrome *c*, *p*-iodonitrotetrazolium violet (Gonzales-Prevatt and Webster 1980; Jakob 1984). The midpoint potentials of the two hemes differ by a remarkable 240 mV: they are +118 mV and −122 mV for the high- and low-potential heme, respectively (Tyree and Webster 1978b).

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Abbreviations. A, absorbance; DEAE-, diethylaminoethyl-; EDTA, ethylenediamine tetraacetate; EPR, electron paramagnetic resonance; HiPIP, high-potential iron protein; SDS, sodium dodecyl sulfate; SOD, superoxide dismutase; VtHb, *Vitreoscilla* hemoglobin; VtmetHb, oxidized *Vitreoscilla* hemoglobin; $VtHbO_2$, oxygenated *Vitreoscilla* hemoglobin

Table 1. Ultraviolet/visible properties of *Vitreoscilla* hemoglobin and derivatives

VtHb derivative		Absorption maxima (nm)	Reference
Oxidized high-spin $Fe(III)_2$	hs VtmetHb	403, 500, 620–640	Tyree and Webster 1978a
Oxidized low-spin $Fe(III)_2$	ls VtmetHb	419, 535–540, 620	Choc et al. 1982
Oxygenated $Fe(II)_2O_2$	$VtHbO_2$	414, 543, 576	Choc et al. 1982
Carbon monoxide $Fe(II)_2(CO)_2$	VtHbCO	419, 535, 566	Webster and Liu 1974
Reduced high-spin $Fe(II)_2$ (dithionite)	hs VtHb	432, 555	Jakob 1984
Reduced oxygenated $[Fe(II)_2]_2O_2$	compound D	418, 548, 576	Tyree and Webster 1979

Earlier investigations on VtHb identified several spectroscopically distinct forms of the heme protein. The electronic absorption maxima and the assignments of these spectroscopic features to different species are summarized in Table 1. The preliminary electronic spectra studies suggested that a low-spin form was present in varying amounts in different preparations. We undertook the combined optical and EPR studies described in this report to examine the putative high- and low-spin forms of ferric VtHb in order to obtain a more detailed picture of the heme iron centers in this interesting bacterial hemoglobin. Effects of basic parameters such as pH, ionic strength and external ligands were also examined. Furthermore, EPR spectra of whole cells as well as of the separated membrane and cytosol fractions of *Vitreoscilla* were investigated. The latter contained the hemoglobin and the NADH-dependent reductase (Gonzales-Prevatt and Webster 1980), and the membrane fraction contained cytochrome *o* (561 564) (Georgiou and Webster 1987b) and cytochrome *bd* (Georgiou and Webster 1987a).

Materials and methods

Materials

The growth of cells, *Vitreoscilla* sp., strain C1, the extraction of VtHb and its purification through the DEAE-cellulose chromatography step were described previously (Webster and Liu 1974). The protein was further purified by chromatography on a Bio-Gel P-100 column (4.5 × 60 cm, Biorad, München). The eluting buffer was 20 mM Tris/HCl pH 7.2, 0.10 M NaCl. Fractions containing VtHb were concentrated by dialysis against Aquacide I or II (Calbiochem, Frankfurt), or pressure filtration through microcollodium bags (Sartorius, Göttingen), then dialyzed against 25% ammonium sulfate in 20 mM Tris/HCl pH 7.6 and processed on a Phenyl-Sepharose CL-4B column (2 × 30 cm, Pharmacia, Freiburg) equilibrated with 25% ammonium sulfate in 20 mM Tris/HCl pH 7.6. The column was washed with 100–200 ml of the ammonium sulfate/Tris/HCl buffer and then eluted with a gradient of ammonium sulfate (25–0%) in 20 mM Tris/HCl pH 7.6. In some cases VtHb was further purified by preparative gel electrophoresis with an LKB 2001 vertical electrophoresis unit using 10% and 17% polyacrylamide gels and a 5% stacking gel (gel size 0.4 × 16 × 18 cm). VtHb (1–1.5 ml) in 20 mM Tris/HCl pH 7.3, 40% sucrose, was applied to each gel. The running buffer was 4 mM Tris/HCl, 40 mM glycine, pH 8.3. The current was fixed at 60 mA when the sample was in the stacking gel, then 90–120 mA after it had penetrated into the separating gel. The electrophoresis was performed at 5–10° C for 10–12 h at which time the front was 1–2 cm from the bottom edge of the gel. The red bands were cut out of the gel, cut into small pieces, and the protein was extracted three times with 0.10 M Tris/HCl, pH 7.3. The membrane and the cytosol fractions were prepared by lysozyme lysis of the cells. After homogenizing a 20% suspension of cells in 50 mM Tris/HCl pH 8.0, 2 mM EDTA was added and the suspension stirred for 10 min. Lysozyme (0.1 mg/ml) and $MgCl_2$ (15 mM) were then added and the suspension was stirred for 20 min. The homogenate was centrifuged at 10 000 × *g* for 15 min and the supernatant centrifuged at 100 000 × *g* for 2 h. The resulting supernatant was used as the cytoplasmic fraction. The pellet was washed with 40 mM phosphate pH 7.3, centrifuged at 100 000 × *g* for 2 h and the resuspended pellet used as the membrane fraction for EPR measurements (Bache et al. 1983).

Methods

Electronic spectra were recorded with a Cary 210 spectrophotometer (Varian, Darmstadt) equipped with a thermostattable cuvette holder. EPR measurements were made on a Bruker B-ER 420 spectrometer equipped with a variable modulation frequency unit, a microwave frequency counter and an NMR gauss meter (Bruker, Karlsruhe). The modulation frequency was 100 kHz and the modulation amplitude 1.0 mT. Samples were frozen in quartz tubes (5.0 ± 0.2 mm outer diameter) in liquid nitrogen. EPR spectra were recorded at 5–12 K using the Helitran LTD-110 C system (Air Products, Allentown). The *g*-values were calculated by measuring the microwave frequency and the magnetic field, calibrated with diphenylpicrylhydrazyl and Mn(II) in MgO. Prominent features of the EPR spectra (extrema, shoulders) obtained for whole cells, the membrane and the cytoplasmic fraction were reported on a *g*-value scale. For sharp and well separated peaks the error will be no more than ± 0.002, when line positions were measured from the field markers on the spectra, whereas for broad features such as the high-field peak of low-spin heme proteins, the error could be as large as ± 0.01. Similarly, if two peaks of similar height overlapped, our measurements may not represent the exact values for each of the individual components, i.e. for EPR spectra of whole cells or preparations of VtHb of low purity. The *g*-values for the different forms of VtHb were assigned on the basis of *g*-values reported in the literature (Harris-Loew 1970; Blumberg and Peisach 1971; Peisach and Blumberg 1971, 1972; Bohan 1977; Walker et al. 1984). Relative amounts of high- and low-spin forms of VtHb were estimated as described (Aasa and Vaenngard 1975; DeVries and Albracht 1979).

Anaerobic samples for EPR and optical measurements were prepared according to Beinert et al. (1978). Anaerobiosis was achieved as described (Riester et al. 1989).

Results

Identification of different spin-state forms of VtHb

Figure 1 shows the low-temperature EPR spectrum of VtHb as isolated according to Webster and Liu (1974). There were strong signals in the low-field region at *g* 6.36, 5.99 and 5.44, a minor signal at 4.34, and weaker

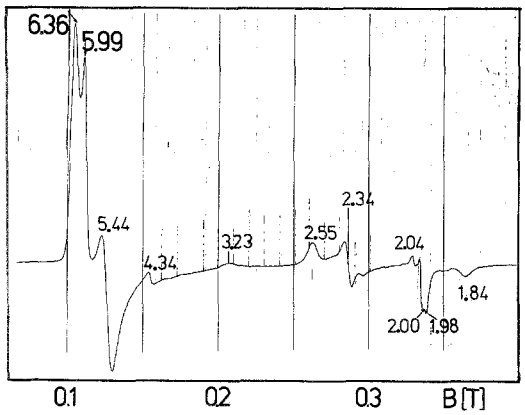

Fig. 1. Electron paramagnetic resonance spectrum of hemoglobin from *Vitreoscilla*. VtHb 0.30 mM in 20 mM Tris/HCl pH 7.3. Microwave frequency 9.370 GHz, modulation frequency 100 kHz, modulation amplitude 1.0 mT, microwave power 1.6 mW, scan rate 1.0 mT s^{-1}, temperature 6 K

signals at higher magnetic field at g 3.23, 2.55, 2.34, 2.00, 1.98 and 1.84. Whereas the first set of lines was assigned to high-spin ferric heme centers with the corresponding high-field lines at g 1.98, the residual lines, with the exception of the signals at g 4.34 and the narrow line at g 2.00, most likely originated from low-spin ferric heme forms (Peisach et al. 1973). The resonance at g 4.34 could result from high-spin ferric-iron-EDTA complexes after cell lysis in the presence of 2 mM EDTA or from denatured iron proteins (Aasa 1970; Bache et al. 1983). Both the lineshape of the low-field resonances in the $g=6$ region and the number of signals at higher magnetic field (region $g=3$-2) varied markedly depending on the purification step and the manipulation of the probe (Jakob 1984). The main fraction obtained after BioGel P-100 chromatography was lowest in low-spin ferric heme components (approximately 10%). In other samples of VtHb prepared by the same procedure the ratio of high-spin to low-spin ferric heme components varied significantly dependent on the growth conditions of *Vitreoscilla*, freezing and storage conditions of the cell and protein material.

Further purification of this BioGel P-100 main fraction by hydrophobic chromatography on a Phenyl-Sepharose column resulted in the separation into fractions enriched in high-spin or low-spin forms of VtHb. The former were actively reduced by the flavoprotein reductase in the presence of NADH whereas the latter were inactive. Earlier, the relative lack of activity observed for these pure fractions of VtHb was attributed to the absence of a required additional component that was presumed to be still present in less pure preparations (Gonzales-Prevatt and Webster 1980).

Preparative gel electrophoresis also led to the enrichment of the high-spin ferric heme components of VtHb. Altogether, four distinct components were separated. The two lesser bands observed during electrophoresis were ascribable to the low-spin and oxygenated forms of VtHb as judged from the electronic spectra of these two fractions. Additionally, two forms of high-spin metVtHb with slightly different EPR signals were separated. Analysis of the low-field part of these EPR spectra revealed several main features: The relative height of the lines in the $g=6$ region varied slightly for each preparation, with two sets of signals at g 6.19, 5.90 (band 1), and g 6.26 and 5.36 (band 2).

An interesting observation came from the effect of 70% ammonium sulfate followed by dialysis against 25% ammonium sulfate. The latter step was employed before binding the heme protein to the Phenyl-Sepharose. Approximately 80% of VtHb was converted to a new low-spin compound with the Soret maximum at 412 nm and dominant resonances at g 3.03, 2.21 and 1.42. This interconversion, high-spin VtmetHb⇄low-spin VtmetHb, was fully reversible: upon decreasing the concentration of ammonium sulfate by a factor of 5 the original EPR spectrum (Fig. 1) and the original electronic spectrum with the Soret maximum at 406 nm and the broad features at 500–550 nm was restored.

Storage of the protein at −20° C over a longer period (1–2 years) resulted mainly in its conversion to low-spin forms as judged from the disappearance of the EPR signals in the $g=6$ region, and the appearance of strong lines at g 3.19, 2.49 and 2.18. On the other hand, no significant changes were observed for samples of VtHb stored at 77 K. Generally, solutions of pure VtHb, in 0.10 M Tris/HCl pH 7.6, could be handled safely at 0–4° C.

Perturbation of the iron centers of VtHb by pH and exogenous ligands

Electronic spectra indicated a transformation of high-spin Fe(III) to low-spin Fe(III) forms as the pH was raised from 7.3 to approximately 10, and at even higher pH there was a marked change in the Soret region (Fig. 2). The corresponding EPR spectra confirmed these observations, i.e. the intensity of the lines in the low-field region decreased. A new signal at $g=3.04$ appeared which disappeared again at pH 11.5 simultaneously with the appearance of a sharp signal at $g=4.28$ indicating the presence of a high-spin Fe(III) site in a highly rhombic environment (Aasa 1970). The signal at $g=4.28$ had a maximum intensity at pH 13. Upon lowering the pH by careful titration of the enzyme sample with dilute HCl, approximately 90% of the original intensity of the EPR signals at low field was recovered. When the protein was titrated with acid to pH 6.5–7.0, the solution of VtHb turned slightly turbid within less than 1 h dependent on the ionic strength. The optical purity index A_{410}/A_{280} decreased from 2.4 to 0.64, most likely because of a loss of the heme cofactor.

The addition (1.0 mM) of either imidazole (Im) or cyanide (CN^-) to a solution of metVTHb in Tris/HCl pH 7.3 resulted in the total conversion of the high-spin Fe(III) heme protein into low-spin Fe(III) forms. The optical and EPR parameters of the different complexes are summarized in Table 2. In contrast, the addition of azide, N_3^- (1.0 mM), had no immediate effect. After long incubation, EPR signals from low-spin Fe(III) centers did appear but, even after a 48-h incubation,

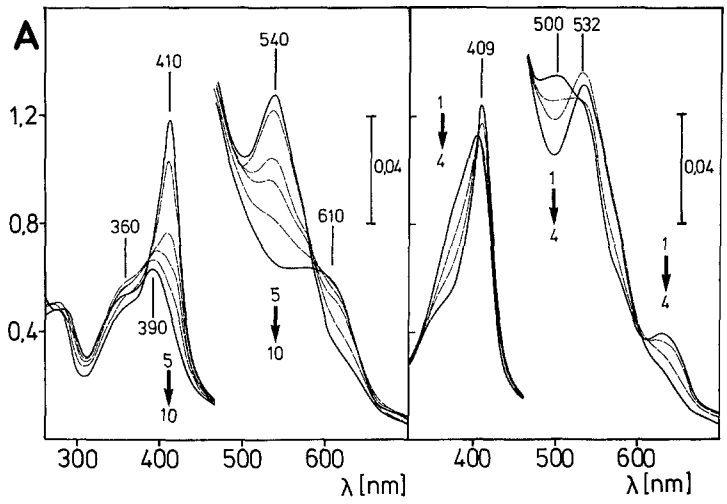

Fig. 2. Spectrophotometric titration of hemoglobin from *Vitreoscilla*. VtHb 73 μM in 10 mM Tris/HCl pH 7.3, 0.10-cm cell; pH was adjusted by addition of 0.10 M NaOH: pH 7.3, 8.3, 9.3, 10.45 (spectra 1-4), 11.0, 11.5, 11.75, 12.0, 12.5, 13.0 (spectra 5-10)

Table 2. Ultraviolet/visible and EPR properties of *Vitreoscilla* methemoglobin and derivatives

metVtHb derivative	Absorption maxima (nm)	EPR signals (g values)
After DEAE-cellulose	406, 492, 538[a], 580[a], 640	6.36, 5.99, 5.44, 1.98 (hs Fe(III)); 2.55, 2.34, 1.84 [ls Fe(III)]
After Phenyl-Sepharose	404, 496, 634	6.38, 6.26, 5.69, 5.56, 1.98 [hs Fe(III)]
Electrophoresis/band 1	405, 500[a], 538, 578, 632	6.19, 5.90, 5.39, 1.98 [hs Fe(III)]
Electrophoresis/band 2	409, 500[a], 538, 578, 630	6.26, 5.93, 5.36, 1.98 [hs Fe(III)]
metVtHb/cyanide	350[a], 419, 542	3.11, 2.22[c], [ls Fe(III)]
metVtHb/imidazole	354[a], 413, 536	2.92, 2.27, 1.55 [ls Fe/III)]
metVtHb/2-mercaptoethanol	422, 542–560[b]	2.47, 2.27, 1.93; 2.39, 2.26, 1.89 [ls Fe(III)]

[a] Weak band or shoulder
[b] Broad band
[c] Not resolved

lines characteristic for high-spin metVtHb were detectable.

In some preparations of VtHb, depending on age and storage conditions, EPR signals were observed at $g=2.43$, 2.24 and 1.91. The electronic spectra of such preparations revealed a new absorption maximum at 533 nm. The g-values were similar to those observed for bacterial low-spin Fe(III) $P\text{-}450_{cam}$ and the high-pH form of ferrihemoglobin A (P-type, $g=2.41$, 2.25, 1.93) suggesting the coordination of cysteine sulfur to the Fe(III) site in VtHb (Bayer et al. 1969; Blumberg and Peisach 1971; Poulos et al. 1985). The addition of 2-mercaptoethanol to VtHb, in Tris/HCl pH 7.6, resulted in a complex spectrum with resonances at $g=2.47$, 2.39, 2.25, 1.93 and 1.89, indicating the presence of two structurally different Fe(III) centers in the low-spin state.

Oxidation-reduction experiments

The addition of oxidizing agents [$Fe(CN)_6^{3-}$, $S_2O_8^{2-}$] to VtHb caused an increase of the EPR signals at low field. Changes in the electronic spectra, viz. disappearance of residual bands at 576 nm and 538 nm and a blue shift of the Soret maximum, were consistent with this being due to the oxidation of residual $VtHbO_2$ in the sample. When solutions of VtHb were treated with NADH in the presence of oxygen and NADH–metVtHb reductase, $VtHbO_2$ was formed immediately (Gonzales-Prevatt and Webster 1980). The EPR spectroscopic investigation of this oxidoreduction showed that only part of the high-spin Fe(III) VtHb reacted. The EPR line at $g=6.36$ decreased considerably (close to 85%). Interestingly, under our conditions, a 100% conversion of the high-spin Fe(III) forms of VtHb into $VtHbO_2$ was never observed. A variable amount of the EPR signal in the $g=6$ region remained detectable, perhaps due to slow autoxidation of $VtHbO_2$. Furthermore, the intensity of the EPR signals from low-spin components of metVtHb did not change in these experiments with NADH and the reductase indicating their lack of reactivity.

When VtHb was treated with NADH and the reductase in the absence of oxygen, there was no significant reduction of the ferric heme components as judged from the EPR and electronic spectra. Subsequent addition of dithionite to the reaction mixture with the exclusion of oxygen resulted in a time-dependent decrease of the EPR signals both in the low- and high-field region. When oxygen was introduced to the dithionite-reduced VtHb the main product of the reaction with oxygen was metVtHb and not $VtHbO_2$, as observed for the reaction of VtHb with $NADH/O_2$. The maximum concentration of $VtHbO_2$ formed in these oxidation-reduction experiments was estimated to be $15\pm5\%$ from the EPR and electronic spectra. The EPR spectrum of the sample which had been treated with dithionite anaerobically and reoxidized by oxygen showed an increase of the resonance at $g=5.99$ relative to the line at $g=6.36$.

EPR-detectable components in whole cells, membranes and cytoplasm of Vitreoscilla

The EPR spectra of intact cells of *Vitreoscilla* showed a variety of intense signals at low and high magnetic field. In the absence of an externally added reducing agent, a strong EPR signal at approximately $g=6$ was observed (Fig. 3). This signal represents several high-spin Fe(III) heme proteins including VtHb. The intensity of the low-field signal was enhanced after addition of an oxidant, such as ferricyanide or persulfate. Furthermore, in the EPR spectra of whole cells a multiline signal around $g=4.3$ was found which originated from the iron-dependent superoxide dismutase (FeSOD). The presence of SOD in *Vitreoscilla* was demonstrated earlier (Orii and Webster 1977). EPR spectra with a comparable pattern and lineshape had been reported for the FeSOD from *Methanobacterium bryantii* (Kirby et al. 1981), *Desulfovibrio desulfuricans* (Hatchikan and Henry 1977) and from the blue-green algae *Plectonema boryanum* (Asada et al. 1975). Furthermore, there were strong signals most certainly associated with Fe-S proteins (Bache et al. 1983). The most prominent features were intense lines at $g=2.08$, 2.05, 2.02, 1.93, 1.88 and 1.81. These features were readily detected in a suspension of whole cells even after addition of the strong oxidants mentioned above, suggesting a rather reducing medium within the cells of *Vitreoscilla*. Consequently, we must expect a major part of VtHb to exist as EPR-silent $VtHbO_2$ or reduced Fe(II)VtHb, as was observed in agreement with the earlier oxidation-reduction experiments using isolated VtHb. Solid sodium dithionite added to an anaerobic suspension of whole cells enhanced the EPR signals at 1.94 and 1.88 supporting the idea that these signals originated from Fe-S proteins (Bache et al. 1983). The very intense and narrow line at $g=2.02$ present in untreated *Vitreoscilla* cells disappeared almost completely after dithionite had been added. Relaxation behaviour, anisotropy and temperatue dependence of this signal is typical for a HiPIP Fe-S protein in the oxidized $[4Fe\text{-}4S]^{3+}$ state, or alternatively [3Fe-4S] clusters (Bache et al. 1983).

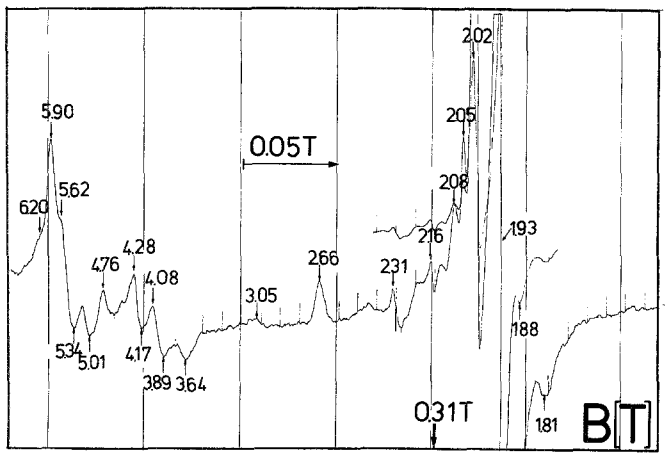

Fig. 3. Electron paramagnetic resonance spectrum of whole cells from *Vitreoscilla* as isolated. Suspension in 50 mM Tris/HCl pH 7.3. Instrument settings as in Fig. 1

A similar picture was found for the membrane fraction. The resonances originating from ferric heme components disappeared after addition of dithionite with concomitant increase of the putative Fe-S EPR signals. In the cytosol prepared by breaking the cells with lysozyme in the presence of EDTA, intense resonances in the $g=6$ region and around $g=4.3$ were also detected. The latter is mainly due to the FeSOD, whereas the strong signals at $g=6$ are most likely a composite of two signals belonging to metVtHb and a catalase. EPR measurements of the partially purified catalase revealed a complex pattern in the $g=6$ region comparable to the pattern observed for the cytoplasmic fraction at low magnetic field.

Discussion

Samples of pure *Vitreoscilla* hemoglobin that were examined both by optical and EPR spectroscopy contained high-spin Fe(III) forms and a variable amount of low-spin Fe(III) components. The signals at low field in the EPR spectrum indicated the presence of both rhombic ($g_x=6.36$, $g_y=5.44$, $g_z=1.98$) and axial ($g_{x,y}=5.99$; $g_z=1.98$) Fe(III) centers (Peisach et al. 1972, 1973; Ruf et al. 1984; Walker et al. 1984). The relative amount of these centers varied in different preparations. Since VtHb is composed of two identical subunits (Tyree and Webster 1978a), this rhombicity could arise by loss of tetragonal symmetry of one or both of the ferric centers within one VtHb molecule. Two bands of VtHb with different ratios of rhombic to axial sites were separated by preparative gel electrophoresis. Preparations of VtHb rich in rhombic Fe(III) centers appeared to be highest in NADH-metVtHb reductase activity. Evidence from rapid kinetic experiments on the binding of CO to VtHb (Orii and Webster 1986) supported a concerted or symmetrical model for the observed cooperative binding (Tyree and Webster 1978a). It is conceivable that upon switching from the T state to the more reactive R state (Perutz 1978, 1980) of VtHb a change in rhombicity of the Fe(III) centers occurs. So far, additional supportive evidence for this intriguing hypothesis is lacking and little is known about the factors that switch the metal sites to a different symmetry of the ligand field. The higher reactivity of the rhombic Fe(III) center towards the reductase may bear no relationship to the reactivity of a comparable Fe(II) form towards CO or oxygen.

The low-spin forms ($g_z=3.23$, $g_{x,y}$ not detected; $g_z=2.55$, $g_y=2.34$, $g_x=1.84$) present in virtually all preparations of pure VtHb were separated from the high-spin forms by hydrophobic chromatography with Phenyl-Sepharose and gel electrophoresis. Obviously, the VtHb molecules rich in low-spin Fe(III) centers possess slightly different physical properties from the VtHb molecules rich in high-spin Fe(III) forms, including hydrophobicity and electrophoretic mobility. Such molecules with low NADH-metVtHb reductase activity

could arise from changes in the chemical environment, e.g. after addition of exogenous ligands such as imidazole or cyanide, changes in pH and ionic strength, or long-term storage at temperatures above −80° C. Although the change from high-spin to low-spin forms of VtHb induced by high pH or high ionic strength is reversible, there is no evidence for the type of equilibrium between high-spin and low-spin forms that is observed for Fe(III) cytochrome *P*-450 in which the concentration of the high-spin form determines the rate of reaction (Guengerich and McDonald 1984).

In several preparations of pure VtHb EPR signals originating from low-spin components were observed at $g_{z,y,x} = 2.43$, 2.24, and 1.91, similar to those observed for low-spin Fe(III) cytochrome *P*-450_{cam} (2.45, 2.26, 1.91) which has a thiolate sulfur from cysteine at the fifth and an oxygen donor atom at the sixth coordination (Lipscomb 1980; Dawson et al. 1982; Poulos et al. 1985). Addition of a nitrogen (imidazole) or a sulfhydryl ligand (propanethiol) caused significant changes in the EPR spectrum of cytochrome *P*-450_{cam} (Dawson et al. 1982). When 2-mercaptoethanol (RSH) was added to VtHb a set of several intense EPR signals from two structurally different low-spin Fe(III) centers were observed characteristic of RS-Fe(III)-N(Im) and H_2O-Fe(III)-SR sites, respectively. The coordination of a thiolate sulfur from cysteine would also explain the EPR signals observed above pH 10. The cysteine residue adjacent to the proximal histidine in VitHb would be a likely ligand candidate for such a conversion (Wakabayashi et al. 1986).

VtHb began to denature and precipitate around pH 6.5 and below, but it was possible to study the properties of the protein by ultraviolet/visible and EPR spectroscopy in basic solution up to pH 13. Even under these conditions approximately 20% of VtHb was in the high-spin state. After readjusting the pH to 7.3, close to 90% of the original VtHb spectral properties were restored. A high recovery of the high-spin components was also obtained when the protein was first treated with ammonium sulfate at high ionic strength and then was dialyzed against a buffer of low ionic strength.

An important finding is that the enzymatic reduction of metVtHb by NADH did not occur in the absence of oxygen. When VtHb was reduced by NADH plus NADH–metVtHb reductase in the presence of oxygen the observed product was $VtHbO_2$. In contrast, no reduction of VtHb occurred in the absence of oxygen. This suggests that the superoxide anion, $O_2^{\cdot -}$, is an intermediate in the formation of $VtHbO_2$; it is known that the reaction of VtHb with superoxide anion generated by xanthine oxidase produced $VtHbO_2$ (Orii and Webster 1977). On the other hand, when VtHb was reduced by dithionite in the absence of oxygen and then exposed to oxygen, the product was metVtHb, and not $VtHbO_2$. This important experimental result explains some of the earlier confusion regarding the functional role of the 'soluble' cytochrome *o* from *Vitreoscilla* (Webster 1987).

The EPR investigations on whole cells confirmed the postulated role of VtHb as a functional hemoglobin. Although there were distinct signals at low magnetic field characteristic for high-spin Fe(III) centers, these were at rather low intensity. This suggests that in the cell VtHb exists primarily in the EPR-silent $VtHbO_2$ form or in the reduced Fe(II) state. A similar picture with respect to VtHb was found for the cytoplasmic fraction. The intensity of the EPR signals in the $g = 6$ region increased after addition of ferricyanide or persulfate. On the other hand, addition of these oxidants to whole cells did not produce major effects on the EPR signals resulting from Fe-S proteins which indicates that there is a strong reducing medium within the cells of *Vitreoscilla*; the intensity of these signals was increased by adding a strong reducing agent such as dithionite.

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