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ALKALINE LOW SPIN FORM OF SULFITE REDUCTASE HEMEPROTEIN SUBUNIT

Lawrence J. Young and Lewis M. Siegel*

Department of Biochemistry, Duke University
and VA Hospital, Durham, NC 27705

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The reversible reduction and reoxidation of *Escherichia coli* sulfite reductase hemeprotein subunit at pH 9.9 produces high and low spin ferric species, the latter with properties distinct from any alkaline low spin yet reported. With virtually no effect on the 298°K optical spectrum, chloride drastically reduces the low spin EPR intensity and produces a high spin conformer pattern like that seen at pH 11. The distribution of g = 5 and g = 2.29 species in the doubly-reduced enzyme is also pH-sensitive.

Upon alkalinization, many ferric hemeproteins exhibit spectral properties which suggest that the Fe coordination number, spin state or axial ligands have changed. It seems settled that the alkaline transitions of methemoglobin and metmyoglobin are due to deprotonation of an axially-coordinated aquo ligand (1-3). Recently a similar phenomenon has been observed for the A_1 and C isozymes of ferric horseradish peroxidase (4) in contrast to earlier reports which argued against formation of the hydroxo complex (5,6). However for oxidized cytochrome c peroxidase (7), chloroperoxidase (8) and myeloperoxidase (9), no compelling evidence has been presented to favor ligation of hydroxide over an endogenous protein ligand. The clarifying accounts of Yonetani and coworkers (7,10) illustrate the difficulty in determining the structural basis of pH-induced spectral effects.

*To whom correspondence should be addressed.

ABBREVIATIONS: CHES - 2-(N-cyclohexylamino) ethane sulfonic acid; D - axial zero field splitting parameter; DMSO - dimethyl sulfoxide; EDTA - ethylene diamine tetra-acetic acid; ENDOR - electron nuclear double resonance; EPR - electron paramagnetic resonance; SiRHP - sulfite reductase heme protein subunit.

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Sulfite reductase hemeprotein subunit when reduced and reoxidized at alkaline pH has spectral and ligand-binding characteristics which suggest that the heme is more exposed than in the oxidized state at neutral pH (11,12). The latter form of the enzyme is believed to possess a penta-coordinate, high spin ferriheme with an open distal binding site. Possible coordination environments of the alkaline form of the enzyme will be discussed.

MATERIALS AND METHODS

E. Coli B cells were grown in bulk on minimal medium (13) by Grain Processing Corp., Muscatine, IA and stored as 2.5 kg cakes at 77°K. Sulfite reductase holoenzyme and hemeprotein subunit were isolated as described previously (11). Following purification the hemeprotein was concentrated to 100-200 μM and stored in standard buffer (0.1 M potassium phosphate plus 100 μM EDTA, pH 7.7) at -20°C. Concentrations were determined with an extinction coefficient of 18.1 mM^{-1} cm^{-1} at 590 nm.

All chemicals were of reagent grade or analytical grade and unless noted otherwise were not purified further. Buffered solutions of KCl and $K_3Fe(CN)_6$ were prepared on ice immediately prior to use. Samples were titrated using aliquots of 1 M KH_2PO_4 or 1 M K_3PO_4 with pH readings taken from a Radiometer PHM-62 standard pH meter attached to a Radiometer C-GK2403 C combined electrode. Cuvettes were thermostatted at 5°C in a PE λ-9 spectrometer that was purged with CO_2-free air.

The anaerobic protocol used was as outlined previously (11) with reduction achieved photochemically using 10 mM EDTA plus either deazaflavin or deazalumiflavin at one-fifth the protein concentration (14). All operations were done at 0-5°C. EPR samples from non-anaerobic, optical experiments were frozen in liquid nitrogen within 30 seconds of completing an optical measurement. A modified version of the Palmer cuvette (15) was used for anaerobic sampling. EPR spectra were taken with a Bruker ER 200D spectrometer at a frequency of 9.47-9.48 GHz, 10 Gauss modulation amplitude and 100 KHz modulation frequency. Temperatures were maintained at 8-14°K by an Air Products Heli-Tran unit.

Ferric low spin and ferrous species were quantified with the method of Aasa and Vanngard (16) while ferric high spins were measured by comparing the area under the g_x feature to that from a sample of resting enzyme (double integral of 0.95 spins/heme vs a Cu-EDTA standard). The accuracy of an EPR quantitation is ± 15% of itself.

RESULTS

The EPR spectrum of doubly-reduced SiRHP at pH 9.9 (Figure 1) has features like those seen when its in standard buffer (17), but differs in the total spins/heme quantitation (0.92 vs 0.82) and the relative amounts of g = 1.94 (0.05 vs 0.03), g = 5 (0.02 vs 0.16) and g = 2.29 (0.85 vs 0.63) species. The reoxidized ferric form (Figure 1) contains four rhombic high spins (Conformers I-IV; g_x = 7.06-6.43; 0.77 spins/heme) and a low spin with g values of 2.56, 2.28, 1.75 (0.19 spins/heme). EPR intensities and the 298°K optical spectrum are unchanged after reac-

tion with excess ferricyanide. The low spin spectrum is abolished with nitrite, cyanide, sulfite and azide; equilibrium measurements show that both spin states disappear together.

33% glycerol diminishes the low spin quantitation to 0.12 spins/heme and concomitantly increases conformer I intensity by the same amount. Optical spectroscopy shows shifts in the maxima of both the α (583 nm to 584 nm) and charge transfer (700 nm to 704 nm) bands and an increase in the intensity of an unshifted Soret band of 6%. 200 mM KCl reduces the low spin to 0.04 spins/heme and lowers the Soret absorptivity by 0.5% without changing the peak maximum. Chloride appears to shift the conformer pattern towards favoring I and III (also seen in pH 11 samples) and produces an additional species with $g_x = 7.28$. We previously suggested (11) that if the value of D for conformer I is not 8 cm^{-1} then the intensity which we measured at 8-14°K will be an underestimate. At 8°K a D of 1 cm^{-1} provides a correction factor of two which gives a spin count close to unity. However even with this change the quantitation in the presence of chloride is still low by 25% (Figure 2) and is not substantially improved by further lowering D. Experimental deficits aside, we have no facile explanation for the shortfall.

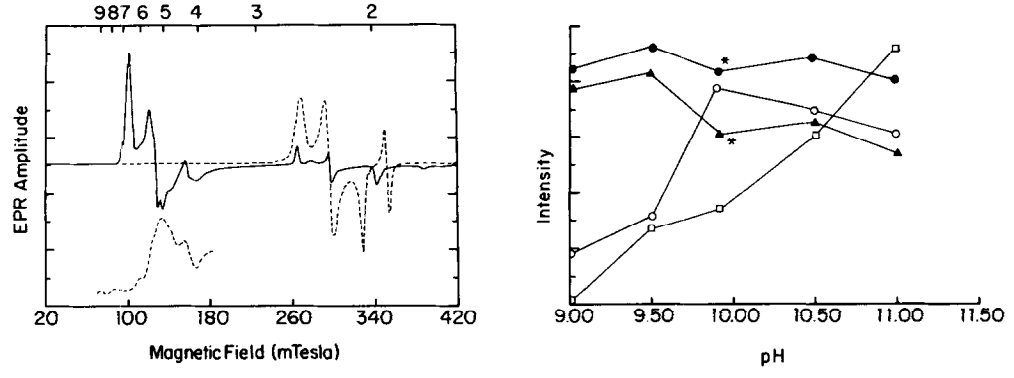

Figure 1: EPR spectra of 321 µM reoxidized (solid) and 375 µM reduced (dashed) heme protein subunit in 0.2 M CHES + 0.01M EDTA pH 9.9. Spectra recorded at 20 mWatt power and a gain of 1.6×10^4 and 2×10^4 for the reduced and reoxidized samples respectively. The gain setting for the expanded trace was 1.6×10^6. In this and the following figure, the intensity scales are in arbitrary units.

Figure 2: EPR pH titration (samples prepared at 5°C) of the reoxidized enzyme from Figure 1 after an eight-fold dilution into the same buffer. EPR samples were taken 30 sec after recording the optical spectrum. Open circle - low spin ferriheme; square - high spin ferriheme, conformer I; triangle - total measured ferriheme spin intensity; closed circle - total corrected ferriheme spin intensity. The top and bottom asterisks correspond to the corrected intensities after adding glycerol to 33% and KCl to 0.2 M respectively. Spectra recorded at 2 mWattt power and gains from $1.25 - 6.3 \times 10^5$.

In Figure 2 are shown the results of titrations with KH_2PO_4 and K_3PO_4, notable features of which are: constancy of the total corrected intensity (vide supra); interconvertibility of high and low spin forms; increased intensity of the most rhombic conformer at higher pH; and decreased low spin intensity above pH 9.9. The low spin titration data does not fit a simple theoretical pH curve, suggesting the presence of multiple processes at the alkaline extreme.

In common with fresh preparations of yeast cytochrome c peroxidase (10) and in contrast to human myeloperoxidase (18), phosphate fails to increase low spin intensity. Rapid freezing in isopentane eliminates the low spin of bovine myeloperoxidase and bovine spleen green hemeprotein (19) but is without effect here. Aging effects are not important since samples prepared from fresh enzyme or that stored for months at -20°C give similar results.

DISCUSSION

When compared to the reduced resting enzyme the distribution of conformeric states of the reduced alkaline enzyme is most like that of the former in standard buffer plus 40% DMSO (17,20). It could well be that both perturbations produce more solvent-exposed or loosely-bound prosthetic groups. The near complete reversibilty of pH effects on the reduced conformers is like what was seen with the reoxidized enzyme (11). The spin state distributions are not the same with the reduced protein being a mix of high and intermediate spin ferrohemes while the reoxidized ferric enzyme contains multiple high spins and a single low spin species which was erroneously attributed in an earlier report (11) to a fraction of reduced enzyme. The low spin is easily saturated and is distinct from a fluoride-induced species ($g_{z,y}$ = 2.7,2.4; 0.3 spins/heme) (11) and the endogenous low spins (g = 2.4, 2.3,1.7) of the holoenzyme (0.2 spins/heme) and SiRHP (0.02 spins/heme) (21). It has unique properties when compared to the alkaline forms of hemoglobin, myoglobin (1-3) and several peroxidases (Table 1) and most closely resembles the yeast cytochrome c peroxidase.

A number of coordinative scenarios can be envisaged for the alkaline enzyme: (1) *All pH 9.9 forms except those with g values of the resting form are 6-coordinate.* This requires that in addition to protonating an hydroxo group, acidi-

TABLE 1

Selected EPR, optical and structural data for peroxidases

Complex[a,b]	EPR signals[c]		Optical[d]		Ligands[e]	
	lo spin	hi spin	Soret	CT	number and type	
ccpf pH 4.3-7.0	2.7,2.2,1.8	6.4,5.3	408	645	6	his/H_2O
					5	his
ccpa pH 4.3	absent	axial	406	616	6	his/H_2O
ccpa pH 7.0	2.7,2.2,1.8	axial	408	642	6	his/H_2O*
					6	his/H_2O
ccpf pH 5.0 + Cl^-	absent	axial	413	640	6	his/Cl^-
ccpf pH 5.0 + F^-	absent	axial	407	620	6	his/F^-
ccpf pH 7.0 + gly	absent	6.4,5.3	nk	nk	5	his
ccp pH 8.5	2.7,2.2,1.8	axial	414	643	6	his/OH^- or prot
mpo pH 4.0-8.0	2.6,2.3,1.8	6.8,5.4	428	620	6	his/?
					6	his/?H_2O
mpo pH 4.0-8.0 + Cl^-	absent	7.0,5.1	434	623	6	his/Cl^-
mpo pH 10.0	2.9,2.2,1.7	present	nk	nk	6	his/OH^- or prot
cpo pH 3.0	2.6,2.3,1.8	7.4,5.3	399	652	6	cys/?
cpo pH 3.0 + Cl^-	2.7,2.3,1.8	7.6,4.1	412	649	6	cys/?
cpo pH 8.2	2.6,2.2,1.8	7.5,4.4	425	639	6	cys/OH^- or prot

a. Abbreviations are: ccp - cytochrome c peroxidase; f - fresh; a - aged; gly - glycerol; mpo - myeloperoxidase; cpo - chloroperoxidase.
b. References are: ccp - 10,22,23; mpo - 9,18,19; cpo - 8,24.
c. All EPR g values are rounded off to two significant digits.
d. Position of Soret and charge transfer band maxima in nm.; nk - not known.
e. His - histidine; cys - cysteine; prot - protein; H_2O* - a water molecule with substantial H-bonding to a distal histidine. Where there are double entries, the top one refers to the low spin species, the bottom one to the high spin species.

fication promotes the dissociation of all distal axial ligands and that on binding to Fe, chloride alters the spin state distribution by replacing H_2O (indirect) or OH^- (direct). The magnitude of the chloride EPR effect is not compatible with a direct reaction and if the indirect reaction reaction took place (either alone or in combination with the direct one) a considerable overlap of "chloride" and "non-chloride" high spin g values would be necessary. Furthermore direct evidence for chloride ligation to the Fe of any hemeprotein is lacking and it must also be explained how chloride and alkalinization to pH 11 produce comparable high spin distributions. (2) *All pH 9.9 high spin complexes are 5-coordinate when chloride is not present and the low spin complex is 6-coordinate with an hydroxo ligand.* Except for chloride binding to a 5-coordinate heme, the hypothesis and the objections to it are as in (1). In both of these cases the protein is almost certainly involved in the pH-induced dissociation of chloride and water either by changing the basicity of a proxi-

mal heme ligand, introducing a distal steric constraint or preventing ligands from having access to the heme pocket. These additional objections to hypotheses (1) and (2) can be abrogated by introducing additional heme and/or protein conformational effects. (3) *All high spins are 5-coordinate, the low spin species has an endogenous protein ligand and the chloride effect is conformational.* This construction has none of the problems associated with the first two. Furthermore, the response to glygerol, the failure of chloride to affect the 298°K optical spectrum and the similarity of V/D and D parameters for the fluoride-induced (0.46,3.36) and alkaline (0.47,4.16) low spins favor this interpretation. (4) *The low spin in (3) is an hydroxo species.*

We cannot presently favor any hypothesis although the third one has a certain simplistic appeal. To date background flourescence (due to destruction of 5-10% of the heme) has prevented use of resonance Raman spectroscopy. $^{1}H/^{2}H$ and/or ^{17}O ENDOR is another possibility and these experiments are now being planned.

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