EPR titration of ovine prostaglandin H synthase with hemin

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To characterize further the prosthetic group of PGH synthase (EC 1.14.99.1), titrations of the apoenzyme with hemin were investigated by EPR. The first hemin bound per polypeptide showed an EPR signal at $g = 6.7$ and 5.3 (rhombicity 9%) and was tentatively assigned to the hemin effective as prosthetic group of PGH synthase. Additional hemin bound showed a less rhombic signal ($g = 6.3$ and 5.8, rhombicity 3%) presumably due to nonspecific hydrophobic binding sites not effective in catalysis.

Prostaglandin H synthase *EPR* *High-spin heme* *Heme binding site* *Cyanide* *Prosthetic group*

1. INTRODUCTION

PGH synthase catalyses the oxidation of arachidonic acid to PGH_2 and represents the initial step of the biosynthesis of prostaglandins, prostacyclin and thromboxanes [1]. The purified enzyme, a polypeptide of about 70 kDa, requires hemin for the reconstitution of enzymatic activity [2–6]. The binding of hemin has been followed by the increase of the Soret band around 410 nm. The reported data have been interpreted as the binding of one hemin [5] or two hemins [2] per polypeptide. The number of hemins, required for the reconstitution of enzymatic activity, is still more uncertain: less than 0.5 hemin (for the bovine enzyme) [4], one hemin [5] or two hemins [2] per polypeptide have been reported. Furthermore it has been suggested that non-heme iron might participate in the catalysis [3,6].

Since number and nature(s) of heme-binding sites are essential for the investigation of the enzymatic mechanism of PGH synthase we used EPR as an alternative spectroscopic method which could better discriminate between different heme-binding sites and further characterize the electronic structure of the heme as prosthetic group of the enzyme.

Abbreviations: PGH, prostaglandin H; PGH_2, 15-hydroxy-9,11-peroxidoprosta-5,13-dienoic acid; SDS, sodium dodecyl sulfate; DPPH, 2,2-diphenyl-1-picrylhydrazyl; DMSO, dimethyl sulfoxide

2. EXPERIMENTAL

PGH synthase was purified as in [5] with slight modifications and showed one band at 72 kDa in SDS–polyacrylamide gel electrophoresis [7]. The preparation contained 0.7 mg detergent (Tween 20) per mg protein [8]. Protein was determined as in [9] with bovine serum albumin as standard. Hemin chloride was from EGA Chemie, Steinheim, and 5 and 10 mM stock solutions in DMSO were prepared immediately before titrations. The concentrations of added ligands are given in mol/mol polypeptide of 72 kDa.

EPR was measured with a Varian E-9 spectrometer operating at 9.2 GHz microwave frequency, 40 mM microwave power and 2.5 mT modulation amplitude. The sample temperature of 90 K was maintained by a cooled gas flow system. The spectrometer was connected to a Data General Nova 3 computer for signal averaging and subtraction of spectra. The g values were determined using magnetic field readings from the Varian 'Fielddial' and DPPH as g marker. The rhombicity R of high-spin was calculated from the splitting Δg at $g = 6$ ($R = \Delta g^* 100/16$) [10]. Optical absorption spec-

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tra of the EPR samples were recorded in the EPR sample tubes with an Aminco DW2 apparatus at room temperature. To reduce the optical pathlength a glass rod of diameter 2.7 mm was inserted in the EPR tubes of inner diameter 3.0 mm. This method yielded as least semi-quantitative optical spectra of the EPR samples [11].

3. RESULTS AND DISCUSSION

The purified PGH synthetase apoprotein contained less than 0.05 mol heme per mol polypeptide as judged by the low absorption at 410 nm and lack of an EPR signal around $g = 6$ (fig.1a) which would both indicate ferric high-spin heme. Spurious EPR signals at $g = 4.3$, indicative of ferric rhombic non-heme iron, were not investigated further since no correlation with enzymatic activity was found.

After addition of hemin various EPR signals around $g = 6$ evolved (fig.1a) in a heterogeneous manner indicating hemin in its high-spin form in different environments. Signals of low-spin hemin were not detected. When hemin was added to buffer alone, no signals at $g = 6$ were observed presumably because of the formation of magnetically coupled dimers in solution. Hence the signals of hemin at $g = 6$ in the presence of protein were assigned to hemin in different binding sites at the protein.

After addition of up to one heme per polypeptide a signal at $g = 6.7$ and 5.3 evolved as was clearly demonstrated by computed EPR difference spectra (fig.2). At these low concentrations of hemin the Soret band of the optical absorption spectrum was observed at 410 nm. At higher concentrations of hemin a signal at $g = 6.3$ and 5.8 appeared with a shoulder at $g = 7.2$. The Soret band concomitantly shifted from 410 nm to lower wavelengths (to 404 nm with 6.1 hemin per polypeptide). The titration curves of the EPR amplitudes at $g = 6.7$ and 6.3 clearly proved a biphasic behavior (fig.3).

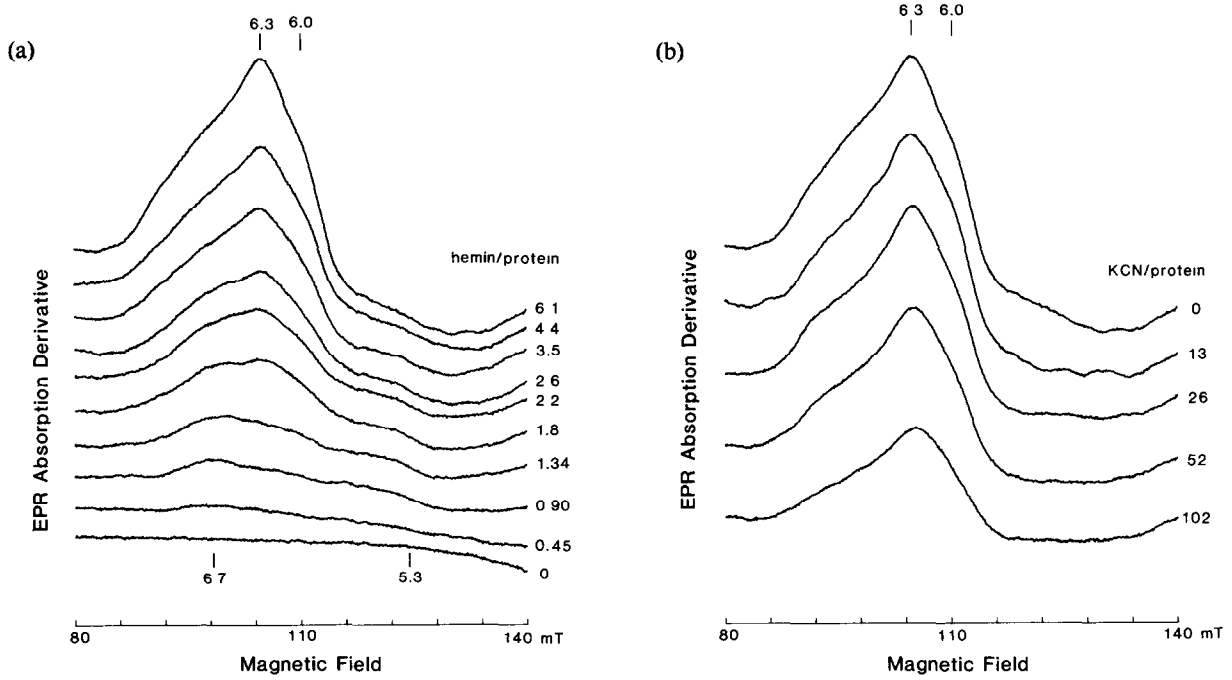

Fig. 1. EPR spectra of the titration of PGH synthase apoprotein with hemin (a) and subsequently with KCN (b). The protein was 44 μM in 0.1 M potassium phosphate buffer (pH 8.1) with 30% glycerol. Hemin was added from the stock solutions in DMSO. KCN was added from a 0.3 M aqueous solution to a sample containing 6.1 mol hemin/mol polypeptide. The ligand concentrations indicated are given in mol/mol polypeptide. The spectra were recorded with a gain of 10^4.

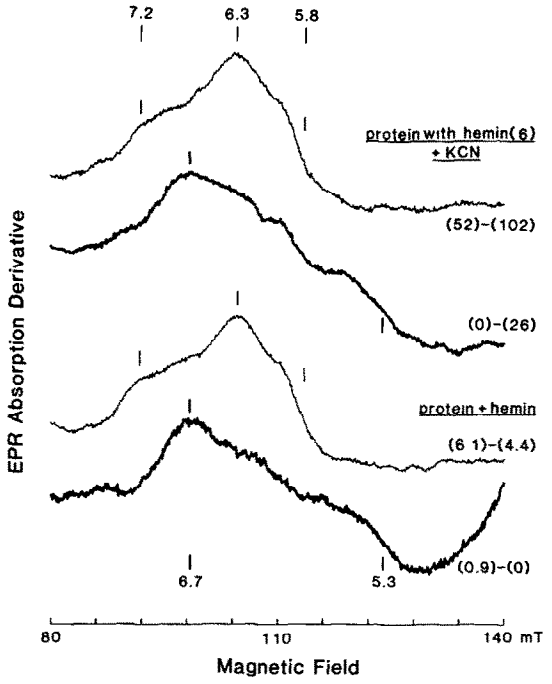

Fig. 2. EPR difference spectra computed from the titrations of fig.1. The numbers in brackets at the curves indicate the spectra of fig.1 from which the differences were computed. The bottom trace is displayed with 4-fold gain compared to fig.1, the other traces with 2-fold gain.

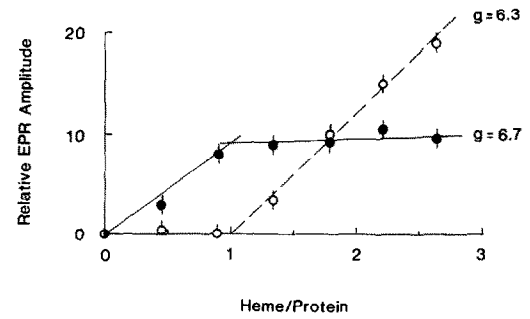

Fig. 3. Dependence of the EPR amplitudes at $g = 6.7$ and 6.3 on added hemin. The amplitudes were taken from the spectra of fig. 1a. The amplitudes were determined against artificial baselines, namely straight lines connecting the points of the spectra 6 mT above or below the corresponding g value. The amplitudes at $g = 6.7$ were corrected for the contributions of the line at $g = 6.3$.

When the sample with a 6-fold excess of hemin was titrated with KCN the signals around $g = 6$ disappeared gradually (fig.1b) with concomitant shift of the Soret band to longer wavelengths (to 425 nm with a 250-fold excess of KCN). The expected signal of low-spin hemin could not be resolved at 90 K. Remarkably, the signal at $g = 6.7$ and 5.3 disappeared first which also had appeared first at the titration with hemin (fig.2). This signal showed a higher deviation from axial symmetry ($R = 9\%$) than the signal at $g = 6.3$ and 5.8 ($R = 3\%$). The signal at $g = 6.7$ and 5.3 corresponded to a Soret band above 410 nm.

These findings together with the recently reported correlation of one bound hemin per polypeptide with fully restored PGH synthase activity [5] provided evidence that one hemin, specifically bound to the more rhombic site, was the prosthetic group of the enzyme. The high rhombicity of this heme was explained by the binding of the heme iron to an axial ligand provided by an amino acid side chain. Such a bond could remove axial symmetry more effectively compared to a distortion of the porphyrin plane induced by the constraint in a protein binding site [10,12].

Since the binding of hemin to serum albumin yielded signals similar to the signal with lower rhombicity (not shown) this signal was assigned to the binding of hemin to hydrophobic sites, either of the protein alone or of micelles of the protein with residual detergent. This unspecific binding may have occurred in earlier heme-binding studies of PGH synthase, especially when Soret bands below 410 nm have been observed [2].

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