Acta Chemica Scandinavica B 34 (1980) 281–284

Soybean Leghemoglobin with Covalently Bound Mesoheme

ULLA PERTTILÄ

Department of Biochemistry, University of Helsinki, Unioninkatu 35, SF-00170 Helsinki 17, Finland

Mesoheme, with 2,4-ethyl side chains, was bound covalently through one of its propionic acid groups to soybean apoleghemoglobin *a*, and the reconstituted protein was purified by isoelectric focusing. The visible absorption spectrum of covalent mesoleghemoglobin in Fe(III) form was mainly of low spin type, while the reduced spectrum was that of unliganded Fe(II) leghemoglobin, *i.e.* of high spin type. A small decrease in p*K* values for imidazole dissociation was observed on covalent binding of heme, indicating a slight tightening of the heme pocket. An attempt was made to bind apoleghemoglobin by covalent linkage to pyrroheme through the lone propionic acid carboxyl at position 7 of the heme ring. Interaction between pyrroheme and apoprotein did not lead to specific covalent binding.

In order to outline the nature of heme–protein interaction in hemoproteins a great number of artificial derivatives with unnatural hemes have been prepared. The 2,4-side chains of porphyrin are vinyls in protoheme, which is the natural prosthetic group of animal and plant globins. The positions 6 and 7 of the protoheme are occupied by propionic acid groups. Interactions between porphyrin and apoprotein, which anchor the porphyrin group relative to movement of the heme iron, have been perturbed by reconstituting hemoproteins with 2,4-modified hemes.[1–6] Structural alterations in reconstituted leghemoglobins have been elucidated by optical absorption and circular dichroism spectroscopy.[7,8] In studies on artificial leghemoglobins, the significance of one propionic acid group on recombination has been established.[9] A role for propionic acid in binding of anionic ligands to Fe(III) leghemoglobin has recently been proposed.[10] In a previous study, the interactions between heme propionic acid groups and soybean apoleghemoglobin have been probed by covalent attachment of heme to protein, followed by the identification of the combination sites in protein.[11] One of the heme propionic acid groups was found to bind covalently to lysine-57(E3) in the E-helix, four residues from the distal histidine residue, whereas in the corresponding myoglobin derivative, the covalent linkage was formed between propionate and lysine CD 3, quite differently from leghemoglobin.[12]

In this study, covalent mesoleghemoglobin was prepared and purified, and spectral and imidazole binding measurements were made to elucidate the environment of the covalently bound mesoheme. In order to identify the bound propionic acid group, covalent binding of apoleghemoglobin with pyrroheme, with only one propionic acid, was attempted. Similarity of the absorption spectra of covalent mesoleghemoglobin and an artificial leghemoglobin reconstituted with pyrroheme XV,[9] initiated an attempt at covalent binding of apoleghemoglobin with this unnatural heme.

EXPERIMENTAL

Purification of covalent mesoleghemoglobin. Covalent mesoleghemoglobin *a* was prepared as previously described.[11] For spectral measurements and ligand binding, the crude preparation was purified by isoelectric focusing in a 110 ml LKB column at a temperature of 4–6 °C.[13] Isoelectric focusing was performed in a sucrose density gradient, the pH range of the carrier ampholytes being 4.5–5.0 (1 %, Ampholine, LKB). The pH values of the resolved fractions were measured at 5 °C with a Radiometer PHM 64 meter calibrated with 0.05 M potassium hydrogen phthalate and Beckman pH 7.0 standard buffer.

Spectral measurements. Absorption spectra and spectrophotometric titrations were performed with a Cary 15 recording spectrophotometer at 22 °C.

0302-4369/80/040281-04$02.50

The absorption spectrum of covalent mesoleghemoglobin in the Fe(III) state was recorded in sodium phosphate (pH 6.4, $\mu=0.1$). Reduction of iron to the Fe(II) form was achieved by addition of sodium dithionite. Mesoleghemoglobin, used as a reference for the spectra, was obtained by the titration method as described previously.[11]

Equilibrium constants for imidazole dissociation were determined spectrophotometrically in a phosphate buffer at pH 6.0.[14] Concentrated solution of imidazole (pH adjusted to 6.0) was successively added to a leghemoglobin solution and the absorption spectrum in the wavelength region 350–450 nm was recorded. The absorbances were corrected for dilution and the dissociation constant was evaluated graphically from the absorbance changes produced by the added amounts of imidazole.[15]

Preparation of pyrroheme sulfuric anhydride. Pyrroporphyrin XV was purchased from ICN·K&K Laboratories, Inc. (Plainview, N.Y., U.S.A.). Iron was inserted by the method described in Falk,[16] and the purity of pyrroheme was checked chromatographically.[17] Pyrroheme sulfuric anhydride was formed by the procedure described previously,[18] but the extraction was omitted because only one type of reaction product was formed.

Combination of pyrroheme with protein. The capacity of apoleghemoglobin to bind pyrroheme and pyrroheme sulfuric anhydride was determined spectrophotometrically. Apoprotein and pyrroheme anhydride were combined in the molar ratio 1:1 in a 0.1 M triethanol–ammonium chloride buffer (pH 7) and the pH was raised to 8.1 with Tris (free base). After 1 h the solution was neutralized and dialyzed. Non-covalently bound heme was removed by the acid-acetone method.[19]

RESULTS

Isoelectric purification and absorption spectra of covalent mesoleghemoglobin. Covalent mesoleghemoglobin yielded one major fraction in the focusing column, which comprised of about 90 % leghemoglobin, (Fig. 1) and had an isoelectric point of 4.8. The remaining coloured material consisted of minor leghemoglobin fractions resulting from the attachment of heme outside the heme pocket.[11] The absorption spectra of covalent mesoleghemoglobin and non-covalent mesoleghemoglobin are presented in Figs. 2 and 3. In the oxidized form, the spectrum of the covalent derivative differed from the non-covalent in being more of low spin type, whereas in the reduced form, both derivatives had high spin spectra.

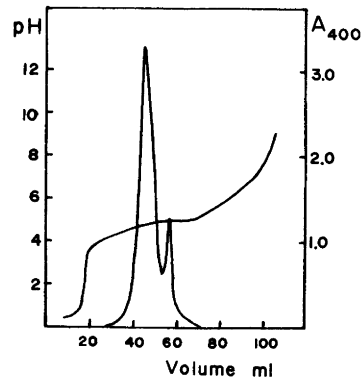

Fig. 1. Purification of covalent mesoleghemoglobin *a* by isoelectric focusing in a sucrose density gradient.

Imidazole binding. Absorbance changes were recorded in the Soret wavelength region, where the wavelength maximum was shifted about 10 nm to red on imidazole binding. The affinity of mesoleghemoglobin for the imidazole ligand was found to be almost identical with that of protoleghemoglobin, since the pK values for imidazole dissociation were 3.01 and 3.04, respectively.[15] The pK value of covalent mesoleghemoglobin was 2.78, indicating slightly diminished affinity.

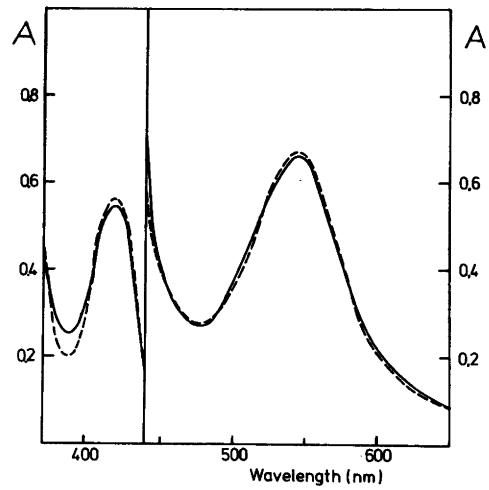

Fig. 2. Soret and visible absorption spectra of mesoleghemoglobin (– – –) and covalent mesoleghemoglobin (——) in Fe(II) state. Buffer was sodium phosphate (pH 6.4, $\mu=0.1$), concentrations were 60 μM and 6 μM in visible and Soret regions, respectively.

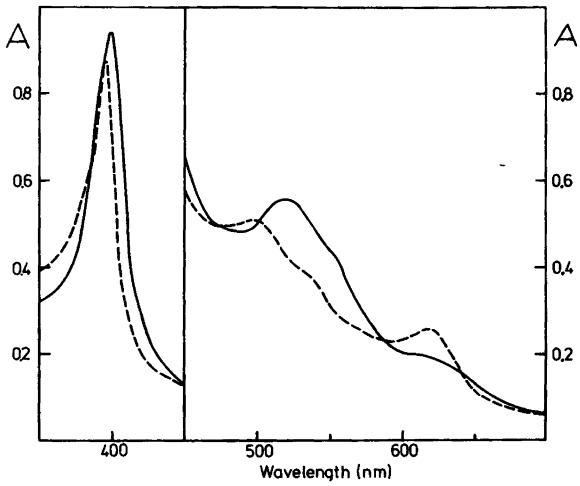

Fig. 3. Soret and visible absorption spectra of mesoleghemoglobin (– – –) and covalent mesoleghemoglobin (———) in Fe(III) state. Buffer and concentrations were the same as in Fig. 2.

Pyrroleghemoglobin. Pyrroheme was observed to bind to apoleghemoglobin with some difficulty, since the titration curve (Fig. 4) shows a rather vague break for an equivalence point with a molar ratio of more than 1:1 for heme to protein. It may be that pyrroheme aggregates very strongly and therefore should be used in extremely dilute solutions. However, extensive dilution is not feasible in this procedure, nor is it possible to use alkaline solutions for disaggregation as the activated carboxyl of propionic acid is quickly disrupted in alkali.

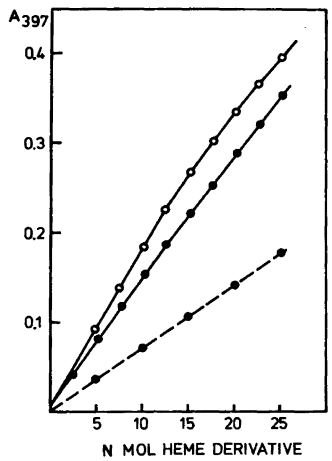

Fig. 4. Spectrophotometric titration of apoleghemoglobin with pyrroheme (●) and pyrroheme sulfuric anhydride (○) at the wavelength 397 nm. 10 nmol apoprotein was in 3 ml of 0.1 M triethanol–ammoniumchloride buffer (pH 7), heme derivatives were added in aliquots of 5 μl. The dotted line indicates blank titration.

When pyrroheme sulfuric anhydride and apoleghemoglobin were combined, considerable brown precipitates were formed on incubation and dialysis of the combination product. This is an indication of an unstable compound, and has been observed before in preparing pyrroleghemoglobin.[9] In the acid-acetone treatment, approximately 90 % of the pyrroheme was transferred to the acetone phase. The remaining covalent pyrroleghemoglobin was hydrolyzed with pronase,[20] and the hydrolysate was subjected to high-voltage paper electrophoresis.[21] The protein hydrolysate was resolved to six hemopeptide bands, indicating non-specific (covalent) binding of pyrroheme, and was not analyzed any further.

DISCUSSION

In preparing the covalent derivative of leghemoglobin, mesoheme was used instead of the naturally occurring prosthetic group, protoheme, because of its better solubility in the course of the anhydride preparation procedure. Even if the substitution of mesoheme for protoheme does not markedly affect the absorption spectrum of leghemoglobin, it gives

rise to small changes in the heme orientation and configuration of the heme pocket.[7,8] Warme and Hager have pointed out the similarity of the absorption spectra of covalent and non-covalent mesomyoglobin in both Fe(II) and Fe(III) states.[12] However, the absorption spectrum of ferric mesoleghemoglobin is changed from mixed spin to nearly low spin type on formation of the covalent linkage between heme and apoprotein. The imidazole nitrogen of the distal histidine appears to be a good candidate for the sixth ligand of the ferric iron in this artificial leghemoglobin. On reduction, a high spin spectrum similar to pentacoordinated ferrous mesoleghemoglobin is obtained. It has been proposed that the distal histidine could not act as the sixth ligand of iron in native ferrous leghemoglobin, as it moves on reduction a considerable distance from the heme.[22]

The substitution of mesoheme for protoheme does not affect the imidazole binding. On formation of the covalent linkage, a slight tightening of the heme pocket is observed as a small decrease in pK for imidazole dissociation, but the flexible mode of the E-helix, which has been repeatedly emphasized, is apparently still preserved.

A tendency of the spin equilibrium towards a low spin state in the spectra of leghemoglobins as compared to those of myoglobins is of interest. The temperature-dependent spin state equilibrium in aquaferric leghemoglobin has been suggested to arise from the flexibility of the protein close to the heme, which enables the distal histidine side chain to move on and off the heme iron.[23,24] In covalent mesoleghemoglobin, a configurational change is deduced to occur in the heme environment, which favours a low spin electronic structure of the ferric iron.

The results in this study show that pyrroheme sulfuric anhydride is incapable of binding covalently in the heme pocket of leghemoglobin. Due to chemical dissimilarity of pyrro and protohemes, pyrroheme–protein interactions are weaker than protoheme–protein interactions. In this case the requirements for the formation of a covalent linkage between pyrroheme and protein obviously are not met.

REFERENCES

1. Tamura, M., Asakura, T. and Yonetani, T. *Biochim. Biophys. Acta 295* (1973) 467.
2. Tamura, M., Woodrow, G. and Yonetani, T. *Biochim. Biophys. Acta 317* (1973) 34.
3. Makino, R. and Yamazaki, R. *J. Biochem. 72* (1972) 655.
4. Makino, R. and Yamazaki, R. *Arch. Biochem. Biophys. 157* (1973) 356.
5. Moon, R., Dill, K. and Richards, J. *Biochemistry 16* (1977) 221.
6. Seybert, D., Moffat, K. and Gibson, Q. *Biochem. Biophys. Res. Commun. 63* (1975) 43.
7. Perttilä, U. and Sievers, G. *Biochim. Biophys. Acta* (1979). *Submitted.*
8. Perttilä, U. and Sievers, G. *Biochem. Biophys. Acta* (1979). *Submitted.*
9. Ellfolk, N. and Sievers, G. *Acta Chem. Scand. 19* (1965) 2409.
10. Trewhella, J., Wright, P. and Appleby, C. A. *Nature 280* (1979) 87.
11. Ellfolk, N. and Perttilä, U. *Biochim. Biophys. Acta 534* (1978) 153.
12. Warme, P. K. and Hager, C. P. *Biochemistry 9* (1970) 4237.
13. Vesterberg, O. and Svensson, H. *Acta Chem. Scand. 20* (1966) 820.
14. Scheler, W., Mohr, P., Pommerening, K. and Behlke, J. *Eur. J. Biochem. 13* (1970) 77.
15. Lehtovaara, P. *Acta Chem. Scand. B 31* (1977) 21.
16. Falk, J. E. *Porphyrins and Metalloporphyrins*, Elsevier, Amsterdam 1964, p. 133.
17. Chu, T. C. and Chu, E. J. H. *J. Biol. Chem. 212* (1955) 1.
18. Warme, P. K. and Hager, C. P. *Biochemistry 9* (1970) 1599.
19. Ellfolk, N. *Acta Chem. Scand. 15* (1961) 545.
20. Taniuchi, H., Anfinsen, C. B. and Sodja, A. *J. Biol. Chem. 242* (1967) 4736.
21. Ambler, R. P. *Biochem. J. 89* (1963) 349.
22. Appleby, C. A. In Marekov, N., Ognyanov, I., Orahovats, A., Tarpanov, V., Trifonov, A. and Vlahov, R., Eds., *Proc. 11th IUPAC Int. Symp. Chem. Natural Products 2* (1978) 30.
23. Appleby, C. A., Blumberg, W. E., Peisach, J., Wittenberg, B. A. and Wittenberg, J. B. *J. Biol. Chem. 251* (1976) 6090.
24. Wright, P. E. and Appleby, C. A. *FEBS Lett. 78* (1977) 61.

Received November 6, 1979.