Oxidative and reductive reactions of sulphhaemoglobin with various reagents correlated with changes in quaternary structure of the protein

Akio TOMODA, Akira KAKIZUKA and Yoshimasa YONEYAMA

Department of Biochemistry, Kanazawa University School of Medicine, Kanazawa, Ishikawa 920, Japan

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The absorption maxima in the Soret region and near 620 nm of sulphhaemoglobin shifted from 419.5 nm to 423 nm and from 623 nm to 619 nm respectively with a decrease in oxygen concentrations of the sulphhaemoglobin solution [101.3, 20.3 and 0 kPa (760, 152 and 0 mmHg) partial pressures]. The major changes in the positions of the absorption maxima occurred drastically at oxygen concentrations between 20.3 kPa and 0 kPa, suggesting that the quaternary structure of sulphhaemoglobin has changed from the R to the T state. Inositol hexaphosphate, a known allosteric effector of haemoglobin, enhanced the shift in peak location. This result supports the view that the shift of the absorption maxima reflects changes in the conformation of sulphhaemoglobin. To investigate the relationship between the conformation of sulphhaemoglobin and the reactivity of the protein with various reagents, we studied the oxidation and reduction reactions of the protein with ascorbic acid, ferricyanide and nitrite under aerobic and anaerobic conditions. The results showed that the rates of oxidation and reduction of ferrous and ferric sulphhaemoglobins with these compounds are associated with the conformation of the protein.

Since the initial report by Hoppe-Seyler (1863), sulphHb has been recognized as a green product of the reaction between Hb and H₂S or some drugs such as phenacetin and the sulphonamides. This protein contains a modified chlorin-type haem, in which one atom of sulphur has been introduced into the cyclic tetrapyrrole system (Nichol *et al.*, 1968). However, little information is available on the physiological properties of sulphHb compared with those of HbA.

Carrico *et al.* (1978*a,b*) prepared almost pure sulphHb and studied O₂ binding to the protein. The results show that the O₂ affinity of the protein is extremely low compared with ferrous HbA, suggesting that sulphHb might correspond to or be similar to the T state of HbA in terms of quaternary structure (Perutz *et al.*, 1974*a*). However, the concept of the R and T states proposed by Perutz (1974*a*) for HbA was not applied to sulphHb. The CO-binding properties of sulphHb have also been measured precisely by Brittain (1981).

We have previously shown that the rate of oxidation and reduction of HbA might be a good indicator for assessing the conformation of the protein (Tomoda *et al.*, 1977; Tomoda & Yoneyama,

1978). However, the oxidation and reduction of sulphHb has not been well studied hitherto, especially in terms of the relationship between the conformation and the reactivity of sulphHb.

In the investigation described in the present paper we studied the absorption spectra of sulphHb in the presence and in the absence of inositol hexaphosphate at different O₂ concentrations (partial pressures). We found that the absorption maxima of the protein shifted in the visible-absorption region according to changes in the O₂ concentrations. Furthermore, we investigated the reactivity of sulphHb with some oxidoreductive reagents under various conditions. On the basis of the results obtained, the conformation of sulphHb was characterized.

Experimental

Preparation of sulphHb

Stripped human HbA, which was prepared by the procedures described previously (Tomoda *et al.*, 1978), was converted into sulphHb by the method of Carrico *et al.* (1978*a*). SulphHb, thus obtained, was applied to a column of CM-Sephadex C-50 equilibrated with 10 mM-potassium phosphate buffer, pH 6.8, and was eluted by pH-

Abbreviation used: Hb, haemoglobin.

gradient solutions prepared from 10 mM-potassium phosphate buffer, pH 6.8, and 40 mM- K_2HPO_4 (Tomoda *et al.*, 1978). Solutions containing sulphHb (90% pure) were obtained at pH 7.7–7.8, and were used for the experiments. The concentrations of sulphHb were determined by the pyridine haemochromogen method (Falk, 1964).

Reduction of met-sulphHb with ascorbic acid

Purified sulphHb was converted into the ferric form (met-sulphHb) with a slight excess of ferricyanide (haem/ferricyanide ratio 1:4), and then was passed through a column (2 cm $\times$ 30 cm long) of Sephadex G-25 (coarse grade) equilibrated with 50 mM-Bistris {2-[bis-(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol}/HCl buffer, pH 7.0, containing 0.1 M-NaCl. The eluates were used for the experiments within 15 min. The reaction was initiated by the addition of 20 μ l of 450 mM-sodium ascorbate to met-sulphHb solution. The increase in absorbance was monitored at 620 nm at 25°C in the presence and in the absence of inositol hexaphosphate.

Measurement of absorption spectra of sulphHb at different concentrations of O_2

The absorption spectra of sulphHb were measured spectrophotometrically between 350 and 650 nm at different concentrations of O_2 [101.3, 20.3 and 0 kPa (760, 152 and 0 mmHg) partial pressures] in Thunberg-type quartz cells. The spectra were also measured in the presence and in the absence of inositol hexaphosphate at different concentrations of O_2 .

Measurement of difference spectra of sulphHb and met-sulphHb induced by the addition of inositol hexaphosphate

The difference spectra between the original solutions containing sulphHb or met-sulphHb (20 μ M in haem) and the solutions containing sulphHb or met-sulphHb (20 μ M in haem) to which various concentrations of inositol hexaphosphate had been added were measured between 500 and 650 nm.

Oxidation of sulphHb

SulphHb was completely reduced by the addition of dithionite under anaerobic conditions in Thunberg-type quartz cells, and then was promptly applied to a column (2 cm $\times$ 30 cm long) of Sephadex G-25 (coarse grade), equilibrated with 50 mM-Bistris/HCl buffer, pH 7.0, containing NaCl, to remove dithionite. The eluates were used within 15 min for the oxidation reaction of sulphHb with ferricyanide and nitrite. In practice, a 1.8 ml solution of ferrous sulphHb (20 μ M in haem) was mixed with or without inositol hexaphosphate. The reaction was initiated by addition of 20 μ l of 5 mM-

$K_3Fe(CN)_6$ or 100 mM- $NaNO_2$ solutions. The decrease in absorbance at 620 nm was monitored at 25°C.

Results

Position of absorption maxima of sulphHb in the presence and in the absence of inositol hexaphosphate at various concentrations of O_2

The positions of absorption maxima of sulphHb in the Soret region and near 620 nm are shown in Fig. 1. With a decrease in the concentration of O_2 from 101.3 kPa to 0 kPa, the absorption maxima shifted from 419.5 to 423 nm and from 623 to 619 nm respectively. The position of absorption maxima changed particularly drastically between aerobic (20.3 kPa) and anaerobic conditions (0 kPa), i.e. from 420 to 423 nm and from 622 to 619 nm. This result suggests that the changes in the absorption maxima may be associated, not with deoxygenation of sulphHb, but with the changes in conformation of the protein. The addition of inositol hexaphosphate, a strong allosteric effector of haemoglobin, caused a marked shift of the position, especially from 419.5 to 422 nm, from 420 to 423 nm and from 423 to 423.5 nm at 101.3, 20.3 and 0 kPa respectively.

Fig. 1. Positions of absorption maxima of sulphHb in the presence and in the absence of inositol hexaphosphate at various concentrations of O_2 .

The absorption maxima in the Soret region (○ and Δ) and near 620 nm (● and ▲) of sulphHb were measured in the presence (Δ and ▲) and in the absence (○ and ●) of inositol hexaphosphate at 101.3, 20.3 and 0 kPa of O_2 .

Fig. 2. Reduction of met-sulphHb (----) and metHbA (—) with ascorbic acid

The changes in concentrations of the reduced form of Hb during the reaction were determined by dividing the changes in absorbance by the millimolar absorption coefficients at 620 nm and 578 nm. The reduction of these haemoglobins was studied in the presence and in the absence of inositol hexaphosphate (P_6 -inositol).

Fig. 3. Inositol hexaphosphate-induced difference spectra of met-sulphHb

The difference spectra induced by the addition of different concentrations of inositol hexaphosphate were monitored between 500 and 650 nm. The numbers 1–6 in the Figure show 10 μ M-, 30 μ M-, 40 μ M-, 150 μ M-, 250 μ M- and 350 μ M-inositol hexaphosphate respectively. The inset shows the changes in absorbance at 620 nm induced by different concentrations of inositol hexaphosphate (P_6 -inositol).

Reduction of met-sulphHb by ascorbic acid

In order to study further the relationship between the conformation of sulphHb and the reactivity of various reagents, we studied the reduction of sulphHb with ascorbic acid. Fig. 2 shows the time course of met-sulphHb reduction by ascorbic acid in the presence and in the absence of inositol hexaphosphate. The reduction of metHb is also shown in Fig. 2 for comparison. The results show that the reduction rates of met-sulphHb by ascorbic acid were much higher than those of metHbA, and that inositol hexaphosphate accelerated the reduction rates of met-sulphHb as well as of metHbA.

Inositol hexaphosphate-induced difference spectra of met-sulphHb

The difference spectra of met-sulphHb induced by inositol hexaphosphate were studied at various concentrations of the organic phosphate (Fig. 3). As the concentration of inositol hexaphosphate increased, the difference spectra of met-sulphHb changed with increase in absorbance near 620 nm. The changes in absorbance occurred up to 350 μ M-inositol hexaphosphate with met-sulphHb (20 μ M in haem). Fig. 3 (inset) shows the changes in absorbance at 620 nm in the presence of various concentrations of inositol hexaphosphate. From the results obtained the dissociation constant of inositol hexaphosphate for met-sulphHb was estimated to be 3×10^{-5} M. These results support the view that inositol hexaphosphate binds met-

sulphHb and modifies the conformation of the protein, as shown clearly in metHbA (Perutz *et al.*, 1974b).

Oxidation of ferrous sulphHb by ferricyanide and nitrite

The time course of oxidation of ferrous sulphHb and ferrous HbA by ferricyanide is shown in Fig. 4(a). The oxidation rates of ferrous sulphHb were much faster (about 20-fold) than those of ferrous HbA. Though inositol hexaphosphate accelerated the oxidation rates of ferrous HbA, the organic phosphate did not alter the oxidation rates of ferrous sulphHb.

On the other hand, the reactions of ferrous sulphHb and ferrous HbA with nitrite showed considerable differences from those with ferricyanide. The oxidation rates of ferrous sulphHb were considerably lower than those of ferrous HbA (Fig. 4b). However, inositol hexaphosphate did not affect the oxidation rates of sulphHb with nitrite, whereas it markedly decreased the oxidation rates of ferrous HbA.

Discussion

It has been shown that Hb takes the two alternative quaternary structures, i.e. the oxy or R form, having a high oxygen affinity, and the deoxy or T form, having a low one (Perutz *et al.*, 1974a; Perutz, 1975). However, there has been little information of the conformation of sulphHb hitherto.

Fig. 4. Oxidation of ferrous sulphHb (----) and HbA (—) with ferricyanide and nitrite in the presence and in the absence of inositol hexaphosphate

The changes in concentrations of the oxidized form of haemoglobin during the reaction were determined by dividing the changes in absorbance by the millimolar absorption coefficients at 620 nm and 578 nm. Inositol hexaphosphate (P_6 -inositol) was present or absent as indicated. (a) Oxidation with ferricyanide; (b) oxidation with nitrite.

Since sulphHb has an extremely low oxygen affinity (Carrico *et al.*, 1978b), it was possible that the protein conforms to the T state, like Hb Kansas (Bonaventura & Riggs, 1968). We observed that the positions of absorption maxima of sulphHb in the Soret region and near 620 nm changed drastically between O_2 partial pressures of 20.3 and 0 kPa in the absence of inositol hexaphosphate, whereas they changed little at O_2 partial pressure between 101.3 and 20.3 kPa. This result suggests that the changes in absorption maxima might accompany a change in quaternary structure from the R to the T state, and that they are not caused simply by the dissociation of O_2 . This view may be supported by the fact that inositol hexaphosphate,

a strong allosteric effector of Hb, caused a marked shift of absorption maxima to longer wavelength in the Soret region. Since the position of the absorption maximum of sulphHb at 0 kPa shifted from 423 nm to 423.5 nm in the presence of inositol hexaphosphate, this fact suggests that deoxysulphHb might be modified by this organic phosphate. The presence of a modified form in human deoxyHb in the presence of inositol hexaphosphate has also been suggested (Tomoda *et al.*, 1978; Mizukoshi *et al.*, 1981).

Our present results show that oxidation rates of sulphHb with ferricyanide are much higher than those of HbA (Fig. 4a). This result may be explained by the concept that sulphHb is probably shifted to the T state under aerobic conditions, though not completely. We previously suggested that the T state of Hb is favoured for the oxidation with ferricyanide rather than the R state, and that the R state is favoured for the oxidation with nitrite rather than the T state (Tomoda *et al.*, 1977; Tomoda & Yoneyama, 1978). From this viewpoint, the lower oxidation rates of sulphHb with nitrite (Fig. 4b) may be also explained by the fact that the protein conforms to the T state under aerobic conditions.

With regard to metHb, Perutz *et al.* (1974b) showed that the quaternary structure of the protein takes the R state and that it changes from the R state to the T state on the binding of inositol hexaphosphate to the protein. It is to be expected that the quaternary structure of met-sulphHb is shifted more to the T state than it is with metHb, because ferrous sulphHb, having an extremely low oxygen affinity, probably takes on the T state much more often than does ferrous HbA. This view may be supported by our results in Fig. 2, i.e. the reduction rates of met-sulphHb with ascorbic acid are much faster than those of metHbA. Our observations are consistent with the results reported by Taketa *et al.* (1980), that the reduction rates of metHb of abnormal Hb Wood ($\beta_{97\text{His} \rightarrow \text{Leu}}$) having an extremely high oxygen affinity and probably taking the R state are much lower than those of normal metHbA, when ascorbic acid is used as a reductant.

Inositol hexaphosphate accelerated the reduction rates of met-sulphHb with ascorbic acid as well as those of metHbA. This result may be explained by the fact that the quaternary structure of met-sulphHb was shifted much more towards the T state by the binding of inositol hexaphosphate to the protein.

Thus our results suggest that different quaternary structures such as the R and the T states might be present in sulphHb and met-sulphHb, similarly to ferrous and ferric haemoglobins. This view should be explored by further examination.

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References

- Bonaventura, J. & Riggs, A. (1968) *J. Biol. Chem.* **242**, 980–991
- Brittain, T. (1981) *Biochim. Biophys. Acta* **673**, 253–258
- Carrico, R. J., Peisach, J. & Alben, J. O. (1978a) *J. Biol. Chem.* **253**, 2386–2391
- Carrico, R. J., Blumberg, W. E. & Peisach, J. (1978b) *J. Biol. Chem.* **253**, 7212–7215
- Falk, J. E. (1964) *Porphyrins and Metalloporphyrins*, pp. 181–182, Elsevier, Amsterdam
- Hoppe-Seyler, F. (1863) *Zentralbl. Med. Wiss.* **1**, 433–434
- Mizukoshi, H., Itoh, M., Matsukawa, M., Mawatari, K. & Yoneyama, Y. (1982) *Biochim. Biophys. Acta* **700**, 143–147
- Nichol, A. W., Hendry, I. & Morell, D. B. (1968) *Biochim. Biophys. Acta* **156**, 97–108
- Perutz, M. F. (1975) *Proc. Int. Conf. Red Cell Metabolism and Function 3rd* 3–20
- Perutz, M. F., Ladner, J. E., Simon, S. R. & Ho, C. (1974a) *Biochemistry* **13**, 2163–2173
- Perutz, M. F., Fersht, A. R., Simon, S. R. & Roberts, G. C. K. (1974b) *Biochemistry* **13**, 2174–2186
- Taketa, F., Matteson, K. J., Chen, J. Y. & Lobnoh, J. A. (1980) *Blood* **55**, 116–118
- Tomoda, A. & Yoneyama, Y. (1978) *Experientia* **35**, 15–16
- Tomoda, A., Matsukawa, S., Takeshita, M. & Yoneyama, Y. (1977) *Biochem. Biophys. Res. Commun.* **74**, 1469–1474
- Tomoda, A., Takeshita, M. & Yoneyama, Y. (1978) *J. Biol. Chem.* **253**, 7415–7419