TURTLE HEMOGLOBIN: EVIDENCE OF A T-STATE OXYHEMOGLOBIN

Sônia R. W. LOURO and G. BEMSKI
Departamento de Física, Pontifícia Universidade Católica, Cx.P. 38071, Rio de Janeiro, RJ, Brasil

Received 27 April 1982

1. Introduction

Hemoglobin presents a great variability to serve the needs of many species. The comparative study of hemoglobins of different species is quite helpful in elucidating properties common to all hemoglobins. The surviving reptiles are the remnants of a group that appeared 110 million years ago and show numerous interesting adaptations. The remarkable adaptation of turtles to anoxic conditions points to the possibility of unusual functional and structural properties of their hemoglobin.

Studies on hemolysates from a number of turtle species [1,2,3,4] showed that in general:

(i) Turtles have multiple hemoglobins (but not polymorphism);
(ii) Turtles hemolysates have lower oxygen affinities than mammalian hemolysates;
(iii) Methemoglobin levels are high, and range from 5–90%;
(iv) Turtle hemoglobin polymerizes, and this process probably involves formation of double molecules linked by one or more disulphide bridges.

This work concerns *Chelonia mydas mydas*, a green, sea turtle. Nitric oxide derivatives were studied. Nitric oxide binds to hemes and is known to be a label sensitive to the quaternary structure of hemoglobin [5–7].

Binding of nitric oxide to hemes produces paramagnetic species with spin 1/2, suitable for EPR studies. The T state is characterized by a well-resolved three-line hyperfine structure in the EPR spectrum, which has been assigned to the α-hemes [7–9].

We show that the EPR spectrum of *C. m. mydas* nitrosylhemoglobin, in the absence of organic phosphates and at pH 7.2, is characteristic of the T quaternary structure. *C. m. mydas* hemoglobin does not exhibit the quaternary-state transition during saturation with nitric oxide. In addition, hybrids of NO and O_2 assume also the T structure. These observations are consistent with the low oxygen affinity and reduced cooperativity exhibited by this hemoglobin. Comparison with the results of NO-hybrids in human hemoglobin A leads to the conclusion that *C. m. mydas* oxyhemoglobin is in the T state.

2. Materials and methods

Chelonia mydas mydas hemoglobin was obtained from fresh hemolysates of erythrocytes washed 3 times with 0.9% NaCl. Red cells were lysed with cold water and removed by centrifugation. The hemoglobin solutions were stored in 50% glycerol, 0.1 mM EDTA, 0.05 M Tris–buffer (pH 8.3) at -10°C (to prevent oxidation to methemoglobin). Prior to experiments necessary amounts of these solutions were dialysed against the proper buffer.

Deoxygenation and reaction with nitric oxide were performed as in [10]. EPR spectra were obtained at -110°C using a Varian V-4500 spectrometer and a Varian F401 temperature controller. Typical experimental conditions were: microwave frequency, 8914 MHz; microwave power, 20 mW; field modulation, 3 G. Optical absorption spectra were recorded with a Cary model 17 D spectrophotometer and fractional oxygen saturations were obtained as in [11]. Electrophoretic analysis was done in cellulose-acetate strips (Cellogel).

3. Results

Contrary to the majority of turtle species electrophoretic analysis for *Chelonia mydas mydas* showed a single hemoglobin component and no dimerization in

Published by Elsevier Biomedical Press
00145793/82/0000–0000/$02.75

Table 1
Oxygen equilibrium properties of *Chelonia mydas mydas* hemoglobin

pH	Buffer	P_{50} (mm Hg)	Hill constant
7.2	Phosphate (0.1 M)	28	1.8
8.0	Phosphate (0.1 M)	17	2.3
8.3	Tris (0.05 M)	6.5	2.7

our hemolysates was detected. Immediately after bleeding, methemoglobin levels were low but rise faster than in human hemoglobin.

The results of oxygen equilibrium studies are shown in table 1. This hemoglobin has a low oxygen affinity and reduced cooperativity. The P_{50} at pH 7.2, phosphate buffer, and 19°C is found to be 28 mm Hg, 3 times higher than the corresponding value for hemoglobin A (9 mm Hg). The Hill coefficient at these conditions is 1.8, while for hemoglobin A it is 2.8. At higher pH-values both oxygen affinity and cooperativity rise. In Tris-buffer the oxygen affinity and cooperativity are higher than in phosphate buffer.

We have used the EPR spectra of nitrosylhemoglobin, Hb_4 (4NO), and hybrids of nitric oxide–deoxy, Hb_4 (NO, 3-deoxy), and nitric oxide–oxy hemoglobin, Hb_4 (NO, 3 O_2), to relate the affinity and cooperativity of turtle hemoglobin to the T–R quaternary structure transition.

Human nitrosylhemoglobin A is essentially in the high-affinity quaternary structure. In contrast to oxyhemoglobin, addition of inositol hexaphosphate switches the allosteric equilibrium towards the T structure [12]. Thus oxygen is more effective in displacing the equilibrium towards the R structure than NO. The change in the quaternary structure is clearly detected by the EPR spectrum of nitric oxide [5–10]. In the T structure the strong *trans*-effect of NO can disrupt the proximal histidine iron bond of α-chains giving rise to a well-resolved 3-line hyperfine spectrum [13, 14].

The EPR spectrum of turtle nitrosylhemoglobin is characteristic of the T structure, even in the absence of IHP (fig. 1). Furthermore, the hybrid Hb_4 (NO, 3-deoxy) at pH 7.2 continues in the T structure when oxygenated to Hb_4 (NO, 3 O_2). These two EPR spectra are shown in fig. 2, together with the analogous spectra for human Hb A.

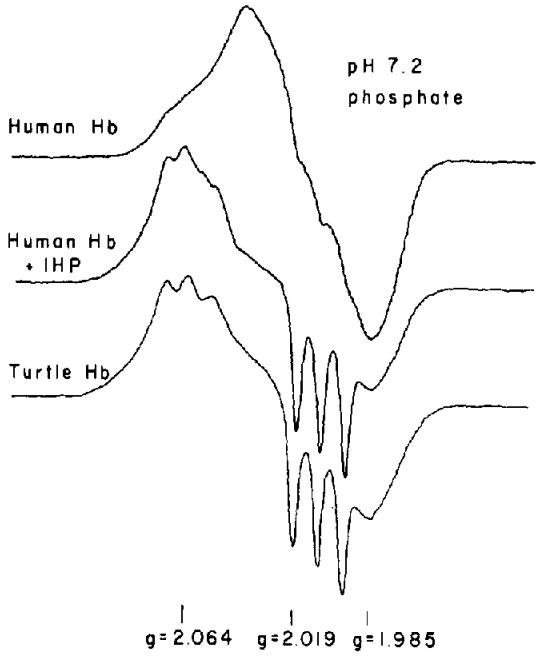

Fig. 1 EPR spectra of nitrosylhemoglobin A with and without IHP and turtle nitrosylhemoglobin without IHP at −110°C (pH 7.2) in 0.1 M phosphate buffer. Hemoglobin was ~1 mM and the molar ratio of IHP to hemoglobin was 2:1.

At higher pH, in phosphate buffer, the EPR spectrum shows, after oxygenation, some displacement towards the T state (fig. 3a). In Tris-buffer, Hb_4 (NO, 3 deoxy) is ~60% R state (fig. 3b), as estimated by comparison with the spectra in [10]. Oxygenation shifts the structure to pure R state.

These EPR results are consistent with the oxygen affinity and cooperativity properties of turtle hemoglobin. The increase in cooperativity is accompanied by the shift of the fully liganded molecule towards the R state.

4. Discussion

The similarity between the EPR spectra of nitric oxide-reacted *C. M. mydas* and human hemoglobin A in R and T states suggests that the immediate heme environment is similar for both hemoglobins. Probably the differences in the tertiary structure, which are responsible for the distinct behaviour concerning the T–R equilibrium, are far from the heme pocket. Any structural interpretation requires, at least, the

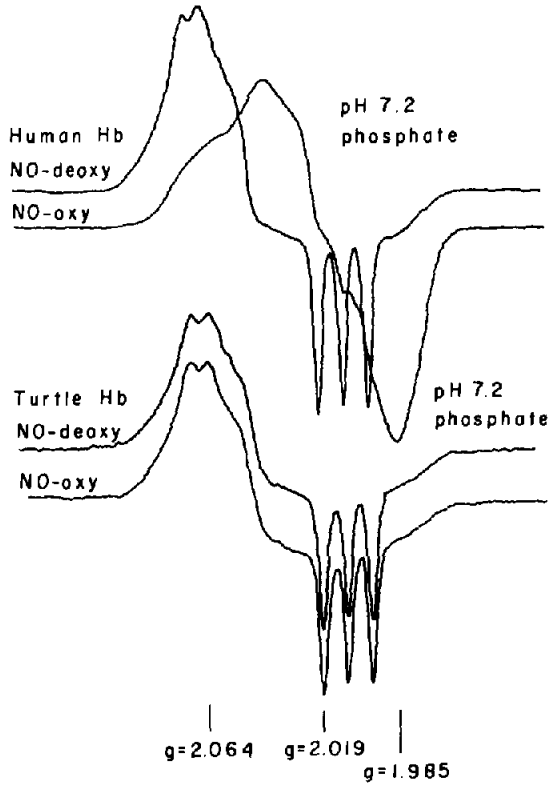

Fig. 2. EPR spectra of the hybrids Hb_4 (NO, 3-deoxy) and Hb_4 (NO, 3 O_2) of human hemoglobin A and turtle hemoglobin at −110°C (pH 7.2) in 0.1 M phosphate buffer.

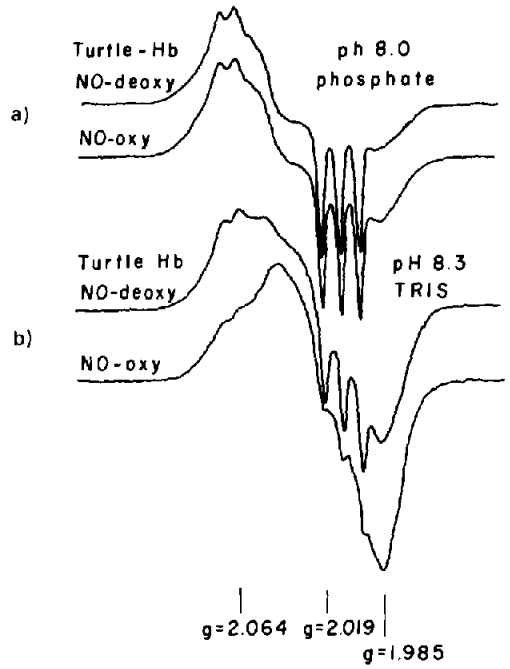

Fig. 3. EPR spectra of the hybrids Hb (NO, 3-deoxy) and Hb_4 (NO, 3 O_2) of turtle hemoglobin at −110°C: (a) pH 8.0 in 0.1 M phosphate buffer; (b) pH 8.3 in 0.05 M Tris.

knowledge of the amino acid sequence, not yet available.

We did not observe any displacement towards the R state after oxygenation of the turtle Hb (NO, 3-deoxy) molecules, at pH 7.2 phosphate buffer. Hence we conclude that in turtle the hybrids Hb_4 (NO, 3 O_2) have a T structure. Conclusion about the quaternary state of turtle oxyhemoglobin is not so direct. Some of the results obtained with NO hybrids of human hemoglobin A [10] can help. EPR measurements of the T–R transition induced by the oxygenation of the human Hb_4 (NO, 3-deoxy) molecules showed that the curve of the fraction of molecules in the R state *vs* partial oxygen pressure is displaced to higher oxygen pressures, when compared to the results predicted by the two-state model. Furthermore, the experimental value of the partial oxygen pressure at 50% R state ($P_{50,R}$) for this sytem is about twice the value for deoxyhemoglobin predicted by the two-state model.

Since in turtle Hb_4 (NO, 3 O_2) no R population was detected, the $P_{50,R}$, if it exists, is very much greater than the oxygen pressure of air (160 mm Hg-opened tonometer). If the effect on $P_{50,R}$ of the ligation of one NO molecule to both human and turtle hemoglobin is the same, the $P_{50,R}$ for oxygenation of turtle deoxyhemoglobin is also $\gg$160 mm Hg. Therefore, we expect that turtle oxygemoglobin is in the T state.

Since the Hill constant at pH 7.2 phosphate buffer is 1.8, it implies that there exists some cooperativity in the T state. Cooperativity within the T structure was also detected in [15].

Acknowledgements

The authors are grateful to Dr Marcel Tabak and Eliane Wajnberg for useful discussions and to Dr J. Moreira Pereira and Dr Adyr N. A. Calado for the electrophoretic measurements. This work was partially supported by the Brazilian Agencies FINEP and CNPq.

References

[1] Sullivan, B. and Riggs, A. (1967) Comp. Biochem. Physiol. 23, 449–458.
[2] Sullivan, B. and Riggs, A. (1967) Comp. Biochem. Physiol. 23, 459–474.
[3] Riggs, A., Sullivan, B. and Agee, J. R. (1964) Biochemistry 51, 1127–1134.
[4] Sullivan, B. (1974) in: Chemical Zoology, vol. 9, ch. 14, Academic Press, New York.
[5] Rein, H., Ristav, O. and Sheler, W. (1972) FEBS Lett. 24, 24–26.
[6] Salhany, J. M., Ogawa, S. and Schulman, R. G. (1975) Biochemistry 14, 2180–2190.
[7] Perutz, M. F., Kilmartin, J. V., Nagai, K., Szabo, A. and Simon, S. F. (1976) Biochemistry 15, 378–387.
[8] Chevion, M., Salhany, J. M. Peisach, J., Castillo, C. L. and Blumberg, W. E. (1977) Isr. J. Chem. 15, 311–317.
[9] Nagai, K., Hori, H., Yoshida, S., Sakamoto, H. and Morimoto, H. (1978) Biochim. Biophys. Acta 532, 17–28.
[10] Louro, S. R. W., Ribeiro, P. C. and Bemski, G. (1981) Biochim. Biophys. Acta 670, 56–63.
[11] Benesch, R., Macduff, G. and Benesch, R. E. (1965) Anal. Biochem. 11, 81–87.
[12] Shulman, R. G., Hopfield, J. J. and Ogawa, S. (1975) Qart. Rev. Biophys. 8, 325–420.
[13] Szabo, A. and Perutz, M. F. (1976) Biochemistry 15, 4427–4428.
[14] Maxwell, J. C. and Caughey, W. S. (1976) Biochemistry 15, 388–396.
[15] Viggiano, G. and Ho, C. (1979) Proc. Natl. Acad. Sci. USA 76, 3673–3677.