THE DEOXYGENATION KINETICS OF HEMOGLOBIN RAINIER ($\alpha_2\beta_2^{145\ Tyr \rightarrow Cys}$)

J. M. Salhany*
Respiratory Protein Laboratory
Veterans Administration Hospital
Gainesville, Florida 32601

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SUMMARY

The deoxygenation rates of hemoglobin Rainier were measured at pH 7.1 in the absence and presence of 2,3-diphosphoglycerate (2,3-DPG). This hemoglobin demonstrated an extremely slow deoxygenation rate which is interpreted as indicating some abnormality in the conformation of the fully liganded protein as well as greatly diminished subunit interaction. The slow deoxygenation rates and the small effect of 2,3-DPG on those rates are consistent with the oxygen equilibrium studies for this hemoglobin and support the view that the deoxy conformation of the protein is unstable, and therefore cannot bind 2,3-DPG strongly.

INTRODUCTION

The tyrosines which normally occupy the HC2 site of the beta chains have been shown by Perutz (1) to perform a role in heme-heme interaction and indirectly in the Bohr effect. In normal deoxyhemoglobin the HC2 tyrosine is found in the pockets between helices F and H. Perutz (1) has proposed that the interaction energy for heme-heme interaction comes mainly from the constraining salt bridges which link the subunits in the deoxy conformation. When oxygen binds, these constraints are broken. The protein trigger for these breaks in the salt bridges has been proposed to be the ejection of tyrosines HC2 from their pockets between helices F and H. Thus in hemoglobin Rainier the absent tyrosines at HC2 would suggest

* Present address and address for reprints: Department of Biophysics, The University of Chicago, Chicago, Illinois 60637.

that cooperativity would be greatly compromised. In addition, the cysteine present at HC2(145)beta in Rainier, has been shown to form a disulfide linkage with the cysteine residue at F9(93)beta. This extra disulfide bridge has the effect of locking the C-terminal region in a fixed position and preventing the salt linkage between the imidazole ring of histidine HC3(146) beta and the gamma-carboxyl of aspartate FG1(94)beta from forming in deoxyhemoglobin Rainier. This latter linkage is responsible for one half of the alkaline Bohr effect. Since the interaction effects between the subunits of hemoglobin as well as the Bohr effect and the effect of organic phosphates are primarily reflected in the ligand off rates of hemoglobin (2,3,4,5), it was elected to investigate the deoxygenation reaction of hemoglobin Rainier for which previous equilibrium studies indicated reduced heme-heme interaction, Bohr effect and effect of 2,3-DPG (6,7,8).

MATERIALS AND METHODS

Blood was drawn in heparin from an individual heterozygous for hemoglobin Rainier in Seattle, Washington. The blood was packed in an ice chest and shipped by air freight to Gainesville, Florida. Plasma and stroma-free hemolysates of the blood were prepared as described before (5). The adult hemoglobin and hemoglobin Rainier were isolated on a CM-Sephadex column (1.8 x 90 cm) using the exact method described by Nagai *et al*. (7). When adult hemoglobin is mentioned in this paper it is the isolated protein from the same individual from whom hemoglobin Rainier was isolated. Each isolated hemoglobin was dialyzed in the cold against a large volume of 0.05 M tris-HCl buffer, pH 7.1 overnight. The deoxygenation reactions were followed at 577 nm using sodium dithionite (1 g/liter) taking care in its preparation. All experiments were performed at $23^{o} \pm 1^{o}$.

RESULTS AND DISCUSSION

The results of the deoxygenation reactions for adult hemoglobin are shown in Fig. 1. They are typical for adult hemoglobin at pH 7.1 and 23^{o}. The values of the overall rate constant calculated as described earlier (5),

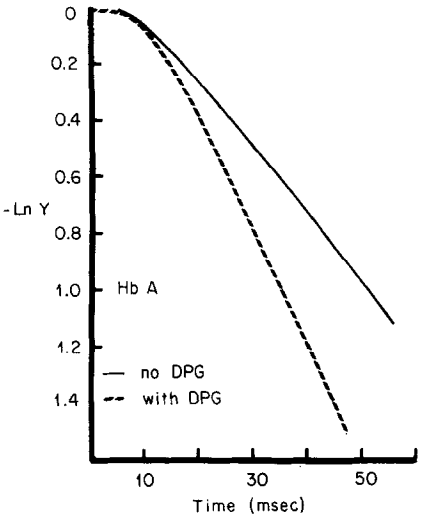

Fig. 1.
The deoxygenation reaction of isolated adult hemoglobin from the individual heterozygous for hemoglobin Rainier. The concentration of hemoglobin before the reaction is 0.02 mM (tetramer). The reaction was followed at 577.0 nm in 0.05 M tris-HCl, pH 7.1 at 23°. The concentration of 2,3-DPG when present was 0.2 mM. The negative of the natural log of the fraction of saturation, Y, is plotted versus time in milliseconds.

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were 22.4 sec^{-1} and 56.8 sec^{-1} respectively for the phosphate free hemoglobin and for the same hemoglobin with a ten to one molar ratio of 2,3-diphosphoglycerate (2,3-DPG) to hemoglobin tetramer present.

When viewing the reaction of oxyhemoglobin with dithionite, the rate of release of the first oxygen molecule as well as the rate of release of the oxygen molecules from the partially saturated intermediates formed during the reaction must be considered. For the dithionite reaction, two limiting cases may be operative: (a) the rates of release from the partially saturated intermediates may be statistically related to the rates of release from fully liganded oxyhemoglobin; (b) the rates of release from all partially saturated intermediates may be larger than that from fully liganded oxyhemoglobin (3). For a hemoglobin which follows the first situation, the overall rate will be equal to $(k_\alpha + k_\beta)/2$ (9). For the second situation, deviation from the curve generated by the non-cooperative case will occur. These considerations are helpful in the interpretation of the kinetic

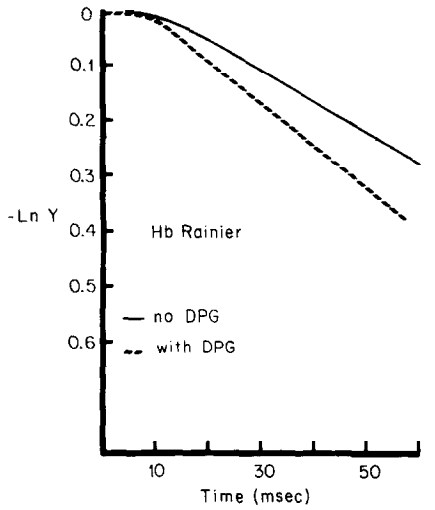

Fig. 2.
The deoxygenation reaction of isolated hemoglobin Rainier. The conditions of hemoglobin and 2,3-DPG concentration and pH, temperature and wavelength are the same as in Fig. 1.

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results for hemoglobin Rainier shown in Fig. 2. From the data of Olson et al. (9) for human hemoglobin at pH 7.0, $(k_{\alpha} + k_{\beta})/2$ is equal to 17.1 sec^{-1}. The values of the overall rate for hemoglobin Rainier in the absence and presence of 2,3-DPG were 6.1 sec^{-1} and 7.8 sec^{-1} respectively. These values are far below those expected for non-cooperative oxygen release from a human hemoglobin, even though the time course indicates greatly diminished cooperativity. Furthermore, the deoxygenation rate is virtually insensitive to 2,3-DPG. These results are quite consistent with the equilibrium measurements reported for Rainier (7,8), which show a considerable reduction in cooperativity (n = 0.99 for stripped Rainier and 1.14 when 2,3-DPG is added), and a significantly reduced effect of 2,3-DPG.

It is of considerable interest to compare these results to those obtained for other mutant and chemically modified hemoglobins. Of particular interest is horse hemoglobin reacted with bis(N-maleimidomethyl)ether (BME)(1,10,11,12) BME reacts with horse oxyhemoglobin by forming intrasubunit, covalent bridges between the SH-group of Cys F9(93)beta, with the second ring reacting at the imidazole of His FG4(97)beta. Thus BME-horse hemoglobin is analogous to

hemoglobin Rainier, in that both have intra beta chain covalent linkages. Kinetic studies of BME horse hemoglobin by Salhany (13), showed that this chemically modified hemoglobin has kinetic properties indicative of a total loss in cooperativity as well as a total loss in the effect of 2,3-DPG. Indeed, the values of the overall rate for BME-horse hemoglobin (14 sec^{-1}) correspond quite closely to the value of $(k_\alpha + k_\beta)/2$ for fully liganded horse hemoglobin measured by Olson et al. (9)(11.2 to 15.1 sec^{-1}). This is significant since BME horse hemoglobin is "locked" in the fully liganded quaternary conformation (1). The extremely low values of the rate constants for Rainier presented herein, seem to indicate that the substitution of cysteine for tyrosine and the formation of a new disulfide linkage between the substituted cysteine and Cys F9(93)beta has a noticeable effect on the functional properties of the hemes in the liganded conformation. Apparently this is not the case for BME horse hemoglobin. Nor is it the case for hemoglobin Bethesda ($\alpha_2\beta_2^{145\ tyr \rightarrow his}$) which has initial rates identical with human adult hemoglobin (14). Furthermore, hemoglobin Bethesda does demonstrate non-cooperative deoxygenation kinetic properties. Thus the deoxygenation kinetics of hemoglobin Bethesda appear analogous to BME horse hemoglobin in that (a) the overall rate constants for both hemoglobins equal the value of $(k_\alpha + k_\beta)/2$ for their respective native hemoglobins and (b) the kinetic time courses indicate non-cooperativity. This differs from hemoglobin Rainier in that, although the deoxygenation time course indicates non-cooperativity, the value of the overall rate constant is very much below $(k_\alpha + k_\beta)/2$. As stated above, this directly suggests that some conformational abnormality exists in the fully liganded quaternary structure, which is influencing the reactivity of the hemes in that conformation. It appears of the utmost interest that an intrasubunit, covalent bridge between the SH-group of F9(93)beta and the non-helical region of His FG4(97)beta results in no functional abnormality of the fully liganded conformation (e.g. BME-horse hemoglobin), whereas an intrasubunit bridge

between Cys F9(93)beta and the substituted cysteine at the HC2 (145) position of the beta chain produces a drastic alteration in the functional properties of the fully liganded conformation (e.g. hemoglobin Rainier).

Lastly, the kinetic results for hemoglobin Rainier support the explanation for the reduction in the effect of 2,3-DPG put forward in the paper by Hayashi and Stamatoyannopoulos (8). They indicated that although hemoglobin Rainier can take a normal quaternary deoxy conformation, as shown by Greer and Perutz (15), the important point, with regard to 2,3-DPG binding, concerns the stability of that conformation. Both the kinetic and equilibrium results support the view that the deoxy quaternary structure of hemoglobin Rainier is unstable, which would appear to be the explanation for the reduced effect of 2,3-DPG.

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REFERENCES

(1) Perutz, M.F., Nature, 228, 726 (1970).
(2) Gibson, J. Biol. Chem., 245, 3285 (1970).
(3) Antonini, E. and Brunori, M., Ann. Rev. Biochem., 39, 977 (1970).
(4) Dalziel, K. and O'Brien, J.R.P., Biochem. J., 78, 236 (1961).
(5) Salhany, J.M., Eliot, R.S. and Mizukami, H., Biochem. Biophys. Res. Commun., 39, 1052 (1970).
(6) Adamson, J., Parer, J.T. and Stamatoyannopoulos, G., J. Clin Invest., 48, 1376 (1969).
(7) Nagai, M., Sugita, Y. and Yoneyama, Y., J. Biol. Chem., 247, 285 (1972).
(8) Hayashi, A. and Stamatoyannopoulos, G., Nature New Biology, 235, 70 (1972).
(9) Olson, J.S., Andersen, M.E. and Gibson, Q.H., J. Biol. Chem., 246, 5919 (1971).
(10) Simon, S.R., Arndt, D.J. and Konigsberg, W.H., J. Mol. Biol., 58, 69 (1971).
(11) Moffat, J.K., J. Mol. Biol., 58, 79 (1971).
(12) Moffat, J.K., Simon, S.R. and Konigsberg, W.H., J. Mol. Biol., 58, 89 (1971).
(13) Salhany, J.M., FEBS Letters 14, 11 (1971).
(14) Olson, J.S. and Gibson, Q.H., J. Biol. Chem. (in press).
(15) Greer, J. and Perutz, M.F., Nature, 230, 261 (1971).