A KINETIC STUDY ON CONFORMATIONAL CHANGE OF DEOXYHEMOGLOBIN INDUCED BY A GROUP OF EFFECTORS

Toyozo Maeda, Keitaro Hiromi* and Shun-ichi Ohnishi

Department of Biophysics and Department of Chemistry*,
Faculty of Science, Kyoto University, Kyoto, Japan

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SUMMARY: The rates of reactions of two different reagents, BTB and DTNB, with hemoglobin were measured to examine the effects of organic phosphates and other compounds on hemoglobin. DPG, ATP and $K_4Fe(CN)_6$, which are known to influence the oxygen affinity, greatly affected the rates for Hb, but showed almost no effects on the rates for HbO_2. By contrast, other compounds with no effects on the oxygen affinity did not change the rates of BTB with Hb and HbO_2. The effectors are considered to induce conformational changes on binding to Hb and such conformational changes are responsible for the control of the oxygen affinity by the effectors.

Benesch, et al. have found that a certain organic phosphates, such as DPG, bound to hemoglobin and remarkably decreased the oxygen affinity of hemoglobin[1-4]. DPG binds to Hb in molar ratio of one DPG per Hb tetramer. It also binds to HbO_2 under a certain conditions, although the binding is much weaker[5,6]. Some thermodynamic studies on this association have also been carried out and a model for the binding has been put forward[3,4].

Such binding would affect the conformation of hemoglobin and the purpose of the present investigation is to detect such conformational changes. We measured the rate of adsorption of

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Abbreviations: Hb, deoxyhemoglobin; HbO_2, oxyhemoglobin; BTB, bromthymol blue; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); DPG, 2,3-diphosphoglyceric acid.

a dye BTB to hemoglobin[7] and also measured the rate of reaction of DTNB (Ellman reagent) with the β93 SH group of hemoglobin[8], to examine the effects of various phosphates and inorganic compounds on the rates. The rate of adsoprtion of BTB to Hb was previously reported by Antonini, et al. to be different from that of HbO_2 and these authors ascribed the difference to difference in the conformations of Hb and HbO_2[7].

MATERIALS AND METHOD: Human adult hemoglobin freshly prepared by toluene hemolysis was rendered phosphate free on a Sephadex G-25 column. This "stripped" hemoglobin was checked to have phosphate content ca. 1% molar ratio by the method of Ames and Dubin[9]. Hemoglobin solutions containing various compounds were transferred to a reservoir syringe of a commercial stopped flow apparatus (Model SPU-1, Yanagimoto Co. Ltd.). Hb, after deoxygenation with nitrogen, was anaerobically transferred to the syringe under N_2 stream. Kinetic measurements were performed in 0.05M Tris-buffer (pH 7.2) at a constant temperature. The reactions of BTB and DTNB with hemoglobin were followed by displaying the optical absorbance at 620 mμ and 460 mμ, respectively, on a memory scope.

RESULTS AND DISCUSSION

Reaction of BTB with hemoglobin. All kinetic curves of hemoglobin with various amounts of compounds were of single phase. The apparent half-time of the reaction with Hb and HbO_2 was 4 msec and 18 msec at 30°C, respectively. Additon of DPG or ATP greatly affected the rate of Hb, while the addition was almost without influence on the rate of HbO_2. The apparent half-time is plotted as a function of DPG or ATP concentration in Fig. 1. The curves tend to saturate as the molar ratio of the effector to Hb in-

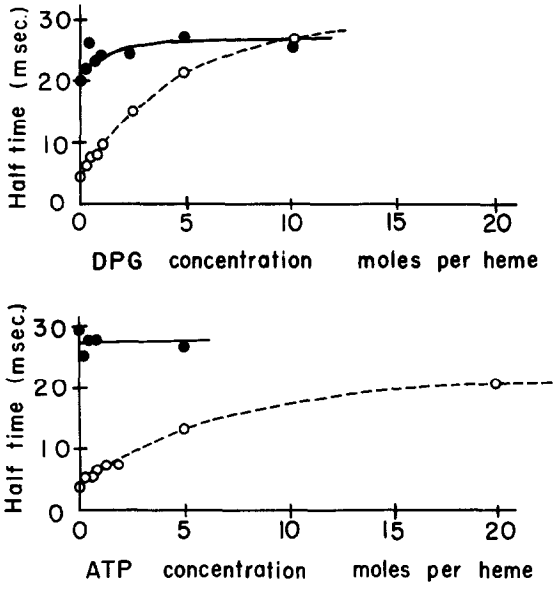

Figure 1. Effect of DPG and ATP on the apparent half-time of the reaction of BTB with HbO_2 (solid line) and Hb (broken line). Concentration: hemoglobin, 1×10^{-4} M; BTB, 5×10^{-5} M. Temperature: 25°C. The values are the average of 5 to 10 experiments.

Table I

Effect of Phosphates and $K_4Fe(CN)_6$ on the Apparent Half-Time of the Reaction of BTB with Hemoglobin.

Concentration in the mixed solution: hemoglobin, 1×10^{-4} M(heme); BTB, 5×10^{-5} M; various compounds, 1×10^{-3} M, otherwise stated. Temperature: 30°C. The values are the average of 5 to 10 experiments. Standard deviation: 2 msec.

Compound	$t_{1/2}$, HbO_2	$t_{1/2}$, Hb
None	18 msec	4 msec
Inorganic phosphate, , 10^{-1} M	18	4
	23	6
Pyrophosphate	20	6
Glycerate-3phosphate	17	4
AMP	18	4
ADP	19	6
ATP	19	12
DPG	18	20
$K_4Fe(CN)_6$, , 1×10^{-2} M	17	12
	18	22

creases. Other organic and inorganic phosphates as well as $K_4Fe(CN)_6$ were also examined for the effects on the rate (see Table I). $K_4Fe(CN)_6$ behaved quite similarly to ATP and DPG, while the other compounds listed in the Table affected the rate only slightly. Comparing these results with those on the oxygen affinity, we observe the parallelism that the compounds affecting the oxygen affinity of hemoglobin yield effects on the rate of BTB adsorption to Hb as well, with the exception of ADP.

Table II

Effect of Inorganic Salts on the Apparent Half-Time of the Reaction of BTB with hemoglobin.

Concentration: the same as given in Table I. Temperature: 25°C.

Compound	$t_{1/2}$, HbO_2	$t_{1/2}$, Hb
None	21 msec	4 msec
ATP		15
DPG		15
NaCl,		5
, 1×10^{-2} M		4
, 5×10^{-2} M		4
, 1×10^{-1} M		4
, 2.5×10^{-1} M	20	4
, 5×10^{-1} M		5
, 1 M	18	5
KCl , 1.3×10^{-1} M	12	4
KI , 1.3×10^{-1} M	19	4
NaI , 1.3×10^{-1} M	6	5

Effects of inorganic salts were examined and the results are summarized in Table II. NaCl showed no effects on the rates of reaction with Hb and HbO_2 in a wide range of concentrations up to 1 M, while this salt is known to give effects on the oxygen affinity at higher concentrations.

Reaction of DTNB with hemoglobin. The kinetic curves of the reaction of DTNB with hemoglobin containing various phosphate

Table III

Effect of Phosphates and $K_4Fe(CN)_6$ on the Initial Rate of the Reaction of DTNB with Hemoglobin.

Concentration in the mixed solution: hemoglobin, 1 X 10^{-4} M; DTNB, 2.5 X 10^{-3} M; various compounds, 1 X 10^{-3} M, otherwise stated. Temperature: 34°C. The values (ΔOD/sec) are the average of 3 to 5 experiments. The optical path: 2 mm.

Compound	Initial rate (X 10^{-4} sec^{-1}) HbO_2	Hb
None	16 + 2	2.5 ± 0.3
Glycerate-3-phosphate	18 ± 1	1.8 ± 0.3
Pyrophosphate	17 ± 2	1.3 ± 0.0
AMP	17 ± 1	1.5 ± 0.3
ATP	20 ± 1	1.0 ± 0.2
DPG	20 ± 0	0.5 ± 0.2
$K_4Fe(CN)_6$	20 ± 1	0.7 ± 0.1

compounds were measured and the obtained initial rates are summarized in Table III. DPG, $K_4Fe(CN)_6$ and ATP decreased the rate of Hb largely but affected the rate of HbO_2 only slightly. The other phosphates also decreased the rate of Hb to lesser extents. The inorganic salts NaCl, KCl, NaI and KI at concentrations of 0.5 M made the rate of Hb about two times faster.

Some changes in the rate of reaction of a reagent with a protein would generally result from alterations in the reaction site and/or its surroundings in the protein, and, therefore can be used as an indirect measure for conformational changes in the protein. In the present kinetic study, we employed two different reagents which probably attack different sites in hemoglobin. DTNB is directed to the site β93 SH group and BTB possibly attaches to sites on the outside of hemoglobin molecule; the nature of BTB-hemoglobin interaction has not yet been well-defined. The results with the two different reagents suggest that the "stripped" Hb and HbO_2 have different conformations and the Hb

is transformed into another conformation on combination with the effectors. Such conformational changes may be responsible for the control of the oxygen affinity of hemoglobin by these effectors.

REFERENCES

1. R. Benesch and R. E. Benesch, Biochem. Biophys. Res. Commun., 26, 162 (1967).
2. R. Benesch, R. E. Benesch and C. I. Yu, Proc. Nat. Acad. Sci., 59, 526 (1968).
3. R. Benesch and R. E. Benesch, Nature, 221, 618 (1969).
4. R. E. Benesch, R. Benesch and C. I. Yu, Biochemistry, 8, 2567 (1969).
5. A. Chanutin and E. Hermann, Arch. Biochem. Biophys., 131, 180 (1969).
6. L. Garby, G. Gerber and C. H. Verdier, European J. Biochem, 10, 110 (1969).
7. E. Antonini, J. Wyman, R. Moretti and A. Rossi-Fanelli, Biochim. Biophys. Acta, 71, 124 (1963).
8. E. J. Neer, W. Konigsberg and G. Guidotti, J. Biol. Chem., 243, 1971 (1968).
9. B. N. Ames and D. T. Dubin, J. Biol. Chem., 235, 769 (1960).