THE EFFECTS OF 2,3-DIPHOSPHOGLYCERATE ON NATIVE AND CHEMICALLY MODIFIED HORSE HEMOGLOBIN

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1. Introduction

The discovery of the effect of 2,3-diphosphoglycerate (2,3-DPG) on hemoglobin [1, 2] has resulted in a number of studies concerning the binding of this molecule to liganded and unliganded hemoglobin [3–6]. The bulk of the evidence suggests that 2,3-DPG does bind to oxyhemoglobin but to a lesser extent than to deoxyhemoglobin. From dissociation kinetic studies [7, 8], it appears that organic phosphates affect at least some partially, if not totally, liganded conformation of hemoglobin. However, 2,3-DPG is believed to stabilize the quaternary structure of the unliganded form of hemoglobin by forming the equivalent of four additional salt bridges between the beta chains [9]. This stabilization of the quaternary structure does not appear to be structurally possible with hemoglobin in the liganded state. Thus, in order to study the functional consequence of 2,3-DPG binding to the totally liganded conformation, it would be convenient to study the effect of 2,3-DPG on a hemoglobin where the quaternary structure is 'locked' in the liganded form independent of the state of ligation. Such a hemoglobin is known to exist when horse hemoglobin is reacted with bis(*N*-maleimidomethyl) ether (BME) [10]. If 2,3-DPG does not affect this hemoglobin then one could conclude that a necessary condition for 2,3-DPG function is at least some partial conformational change in hemoglobin. The conclusions from the studies reported here support the above supposition.

2. Materials and methods

Horse blood was drawn and placed in sodium citrate. The hemoglobin was prepared as described earlier to yield a hemolysate free of organic phosphates [7]. Part of this hemoglobin was dialyzed against 0.05 M trishydroxymethylaminomethane-HCl (THAM) buffer at pH 7.0 and 4°. The second portion was reacted with BME exactly as described by Simon and Konigsberg [11]. Separation of the hemoglobin containing two moles of BME was accomplished on a 2 X 40 cm column of Biorex 70 (BioRad Co.) employing a discontinuous sodium phosphate gradient from 0.055 M to 0.2 M at pH 6.8. This isolated hemoglobin was then dialyzed against 0.05 M THAM, pH 7.0 at 4° and used for the experiments.

The hemoglobin-oxygen equilibrium studies were performed exactly as by Keys et al. [12], at 23°. The oxygen dissociation kinetics were performed as described before [7]. Four hemoglobin preparations were studied with these techniques. These included purified, native horse hemoglobin with and without 0.2 mM 2,3-DPG, as well as BME-reacted horse hemoglobin with and without the same level of 2,3-DPG. All studies were performed on samples containing 0.02 mM concentrations of hemoglobin tetramer in 0.05 M THAM buffer at pH 7.0.

3. Results

The results of the hemoglobin-oxygen equilibrium experiments are presented in fig. 1 with values for P_{50} (half saturation point) and Hill's constant, n, in table 1. It is noted that BME-reacted horse hemoglobin demon-

Table 1
Half saturation point (P_{50}) and Hill's constant, n, for native horse and BME-reacted horse hemoglobin. Also, the overall oxygen dissociation constant (k_d in sec^{-1}) calculated between 85 and 40 percent saturation. From data of figs. 1 and 2.

Hemoglobin	P_{50} (mm Hg)	n	k_d (sec^{-1})
Native horse	5.10	2.4	20.4
Native horse plus 2,3-DPG	7.10	2.9	28.4
BME-horse	1.46	1.0	14.2
BME-horse plus 2,3-DPG	1.48	1.1	13.6

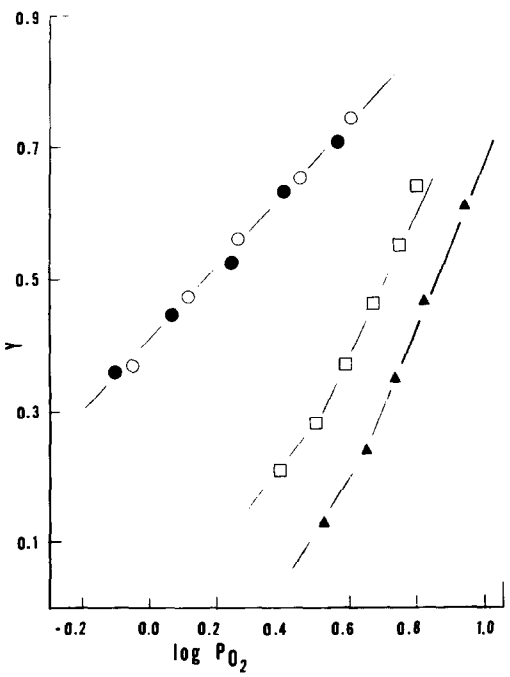

Fig. 1. Equilibrium studies for 0.02 mM native horse hemoglobin and BME-reacted horse hemoglobin in the absence of 2,3-DPG and with 0.2 mM 2,3-DPG in 0.05 M THAM, pH 7.0, at 23°. Plotted as fraction of saturation, Y, vs. log P_{O_2}. ○: BME-horse hemoglobin; ●: BME-horse hemoglobin with 2,3-DPG. □: native horse hemoglobin; ▲: native horse hemoglobin with 2,3-DPG.

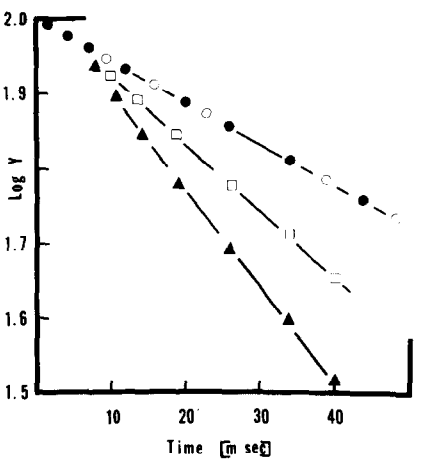

Fig. 2. Time course of deoxygenation for 0.02 mM native horse and BME-reacted horse hemoglobin with and without 2,3-DPG in 0.05 M THAM buffer, pH 7.0, at 23°. Plotted as log of the percent saturation vs. time in msec. Same symbols as in fig. 1.

strates no cooperativity as indicated by n being equal to one. Furthermore, no effect of 2,3-DPG was observed on this hemoglobin. Native horse hemoglobin, however, demonstrated a normal value of n in each case, and a lower oxygen affinity when 2,3-DPG was present. As can be seen from fig. 2 and table 1, the results of the kinetic experiments are in good agreement with the equilibrium data. The high affinity BME-horse hemoglobin demonstrated a slower deoxygenation time course compared to the native hemoglobin. This seems in accord with its higher affinity. Interesting also, was that no lag period was observed for BME-horse hemoglobin, whereas one was consistently demonstrated for the native protein. Furthermore, 2,3-DPG did not affect the rate of deoxygenation for the BME-reacted hemoglobin whereas it did for native hemoglobin, after the lag period. It has been pointed out that this invariance in the lag time in the presence of 2,3-DPG implies and is directly related to an invariant rate constant for the dissociation of the first oxygen molecule from fully liganded hemoglobin [8].

4. Discussion

In a series of three papers, Simon, Arndt, Konigsberg and Moffat [13–15], define quite clearly the structural and functional properties of BME-horse oxyhemoglobin as well as other chemically modified horse hemoglobins. It is demonstrated that one BME molecule binds per beta chain, forming an intrachain bridge between Cys F9(93) beta and His FG4 (97) beta. As mentioned, the quaternary structure is that of the fully liganded native protein. An important tertiary structural change occurs with the displacement of Tyr HC2(145) beta from its original position in native oxyhemoglobin to a new position in the internal (central) cavity, immobilizing the *C*-terminus of each beta chain. It is also noted that BME-oxyhemoglobin has no Bohr effect at all.

With these considerations we can attribute the absence of any effect of 2,3-DPG on BME-reacted horse hemoglobin to two possible causes. These include the 'locking' of the quaternary structure in the liganded conformation and the immobilization of the *C*-terminus of the beta chains. It has been clearly demonstrated that HC3(146) beta histidine accounts for one-half of the alkaline Bohr effect [16]. Furthermore, one of the proposed binding sites for 2,3-DPG is the H21(143) beta histidine [17]. Earlier, it was suggested that the effect of 2,3-DPG is either directly or indirectly linked to the alkaline Bohr effect, since the maximal effect of 2,3-DPG on the kinetics of deoxygenation was observed near pH 7.0 [7]. The following indirect linkage of the kinetics of 2,3-DPG binding to the alkaline Bohr effect may be suggested. Rapid, Bohr proton-linked, tertiary structural changes which are thought to occur at the *C*-terminal ends of the beta chains [18], could make the H21(143) beta histidines first available for 2,3-DPG binding with the other sites thought to be involved [NA1(1) beta valines and EF6(82) beta lysines], becoming available later in the course of deoxygenation (after the quaternary flip to the deoxy form). If this is the basic kinetic mechanism for the proposed indirect linkage, then it would seem that the immobilization of the *C*-terminal end of the beta chains and the absence of a Bohr effect significantly contributes to the lack of any effect of 2,3-DPG on BME reacted horse hemoglobin.

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