BINDING OF 2,3-DIPHOSPHOGLYCERATE TO NORMAL AND ABNORMAL OXYHEMOGLOBIN

D.DIEDERICH, A.DIEDERICH, J.CARRERAS, S.CHARACHE and S.GRISOLIA

Departments of Biochemistry and Medicine, University of Kansas Medical Center, Kansas City, Kansas, and Department of Medicine, Johns Hopkins Medical Institue, Baltimore, Maryland, USA

Received 24 March 1970

1. Introduction

It has recently been shown that oxyhemoglobin extensively binds 2,3-diphosphoglycerate (DPG) and that a portion of this cannot be removed by Sephadex filtration [1] and remains bound to tryptic peptides after digestion of hemoglobin with trypsin [2]. This firmly bound DPG will be referred to as residual DPG (R-DPG).

This paper illustrates that the R-DPG, present in different proportions in normal and abnormal hemoglobins, can be removed by DEAE Sephadex chromatography. However, on adding DPG to DPG free hemoglobin, a similar amount of DPG again becomes firmly bound, i.e., it cannot be separated by Sephadex filtration. In addition this paper illustrates differences in DPG binding by a number of hemoglobins which have marked differences in affinity for oxygen. While in all cases there is binding of DPG to oxyhemoglobin, the binding is not directly related to the oxygen binding capacity of the hemoglobins.

2. Materials and methods

The hemoglobins were purified essentially as described by Huisman and Dozy [3]. Hemolysates, containing 100–150 mg of Hb, were chromatographed on 1 × 25 cm columns of DEAE Sephadex A50. Elution was carried out with a linear pH gradient (500 ml of 0.05 M tris-Cl, pH 8.2 in mixing vessel and 500 ml of 0.05 M tris-Cl, pH 7.2 in the reservoir vessel) at a flow rate of 200 ml/hr. In some cases after the completion of the pH gradient, the DPG was eluted with 0.5 M NaCl–0.5 N HCl. The tubes containing hemoglobin were pooled and concentrated by ultrafiltration (Diaflo; *under oxygen*) to about 1–2 mM Hb. To remove the tris buffer, samples containing 100–150 mg of Hb were passed through 1.2 × 95 cm Sephadex G-25 columns equilibrated with 0.1 M NaCl. The tubes containing the bulk of the Hb were pooled and concentrated to 0.8–1.0 mM by ultrafiltration. DPG binding by hemoglobin was studied as previously described [2].

All column experiments were conducted at 24° using oxyhemoglobin. However, the tubes collected during equilibration experiments were kept at 4° until analyzed; hemoglobin specimens used in these studies were stored at −20°. The cyclohexylammonium salt of DPG (Boehringer) was converted to the potassium salt by percolation through a column of Dowex 50 resin, and then adjusted to pH 7.0 with KOH. DPG was assayed by an automated technique [4]; ^{32}P was measured as previously described [2].

3. Results and discussion

Experiments were carried out to check completeness of DPG removal from hemoglobin by DEAE Sephadex chromatography. One ml samples of 1.5 mM hemoglobin solutions which had been freed of DPG by DEAE Sephadex chromatography were mixed with 50 µl of 30 mM DPG (∿ 1.5 mM final

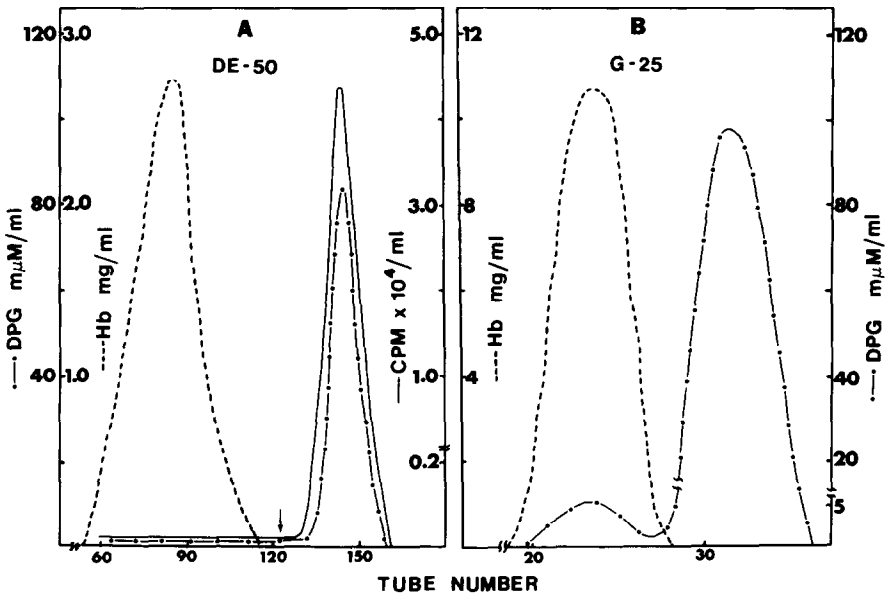

Fig. 1A. See methods for details. Arrow denotes start of 0.5 M NaCl–0.5 N HCl elution of column; three ml aliquots collected.
B. 1 ml samples of 1.5 mM Hb, freed of DPG on DEAE Sephadex column, were made 1.5 mM in DPG at pH 7.0 and then passed through standard Sephadex G-25 column. Two ml aliquots were collected.

DPG) at pH 7.0 and then passed through the standard Sephadex G-25 fine columns and analyzed for hemoglobin and DPG content. In addition, 1.5 ml samples of hemolysates containing 1.5 mM hemoglobin were passed through the Sephadex G-25 columns and shown to contain R-DPG [1]. The hemoglobin pool from this column was chromatographed on a DEAE column as outlined earlier in an attempt to remove the remaining R-DPG.

Fig. 1A illustrates complete removal of DPG from hemoglobin and the subsequent elution of a portion of the adsorbed DPG from a column of Sephadex DEAE 50. This behavior has been observed with all (normal and abnormal) hemoglobins tested. In all cases no, or negligible, DPG remains bound to hemoglobin. Fig. 1B illustrates the incompleteness of DPG removal by gel filtration when DPG was added to hemoglobin initially freed of DPG by DEAE Sephadex chromatography. *In other words, the "hemoglobins" behave insofar as "residual DPG" on filtration through Sephadex as it did before treatment with DEAE Sephadex.* This point is further illustrated below with several types of hemoglobin.

Binding of DPG to hemoglobin varies with some types of hemoglobin [5]. Fig. 2 typifies binding of DPG by several hemoglobins as tested by equilibrium filtration on Sephadex columns. It is of much interest to find out if there is any correlation between binding, residual binding and oxygen affinity of several hemoglobins. A summary of binding for several hemoglobins is shown in table 1. While in all cases there is binding of DPG to oxyhemoglobin, it *does not appear to be directly related* to the reported whole blood oxygen binding capacities of the abnormal hemoglobins. On the other hand, R-DPG may be so related. A discrepancy in oxygen affinity for whole blood and that of hemolysates or purified hemoglobins has been noted for several (abnormal) hemoglobins. Cord blood displays increased oxygen affinity in comparison to normal adult blood. However, solutions of hemoglobin A and hemoglobin F, dialyzed against buffered solutions at pH 7.4, have the same oxygen affinity [6]. Blood of patients with sickle cell anemia exhibits decreased affinity for oxygen while the oxygen affinity of hemoglobin S, as well as hemolysates of hemoglobin SS red cells, is the same as that of hemoglobin A [7]. Previous studies have shown that DPG binding by hemoglobin depends exceedingly on the ionic strength

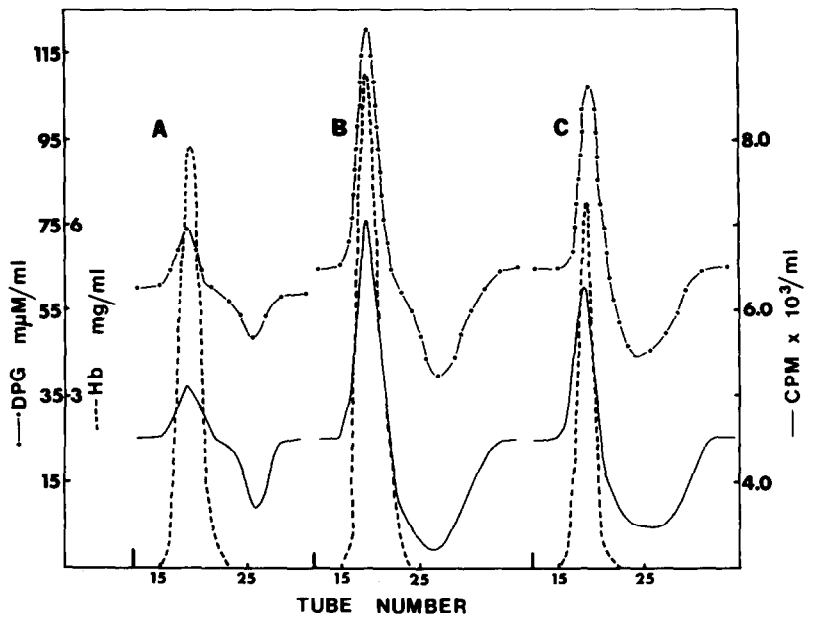

Fig. 2. Matched 0.5 X 120 cm columns of Sephadex G-25 Superfine were equilibrated simultaneously, using a multichannel pump, with 0.06 mM DPG containing $D^{32}PG$ (1 X 10^4 CPM/ml) in 0.02 M NaCl, pH 7.0. One-half ml aliquots of 0.4 mM Hb solutions containing 0.06 mM DPG and 0.02 M NaCl, pH 7.0, were passed through each column at a flow rate of 7 ml/hr. One-half ml aliquot were collected and analyzed for Hb, DPG and radioactivity. Profile A represents data for Hb F, B for Hb Zurich and C for Hb Chesapeake.

Table 1
Summary of DPG binding by several hemoglobins[a].

Type Hb	Residual DPG	DPG binding[b]
AA	0.060 (0.028)[c]	1.03
AA	0.059 (0.031)[c]	1.43
AA	0.056 (0.028)[c]	
Zurich[d]	–	1.02
Chesapeake[d]	–	1.14
J.Baltimore[d]	–	1.09
Fetal	0.097	0.10
Kansas	0.106	1.21
SS	0.018	0.41

[a] All data represent moles of DPG/mole of hemoglobin.
[b] Conditions were as outlined previously [2].
[c] The figures in parentheses represent values obtained by restoring equimolar concentration of DPG to hemoglobin specimens (freed of DPG by DEAE Sephadex chromatography) and then passing the specimens through Sephadex G-25 columns (conditions detailed in legend to fig. 1B).
[d] These hemoglobins were chromatographically purified on DEAE Sephadex column; R-DPG is removed by column step.

of media as well as pH [5]. Therefore, the data in table 1 represents comparative rather than absolute data. Nevertheless, in many experiments the decreased DPG binding of hemoglobins F and SS have been repeatedly observed. As noted above, fetal blood exhibits *increased* while sickle cell blood exhibits *decreased* oxygen affinity. Moreover, hemoglobins Zurich and Chesapeake demonstrated DPG binding equal to or, in some experiments, greater than Hb A [5], and yet both have an increased affinity for oxygen. These findings, together with lack of agreement between the concentration of DPG in red cells and oxygen affinity [7], necessitate further clarification of the binding of DPG to hemoglobin before direct physiological and pathological relationships between DPG and hemoglobin can be understood. It will be important to determine relative binding of these hemoglobins in the reduced form. It seems likely that extension of the present studies, particularly of R-DPG, may shed some light on the physiological and pathological roles of DPG in relation to hemoglobin structure and function.

Acknowledgements

This research was supported by PHS, Grants AMO1855, AM13119, 1-F3-GM-23, 208-01, 1 F05, TW01388-01, the American Heart Association, and the Kansas Heart Association. J.Carreras (on leave of absence from the University of Barcelona) is an International Fellow of the US Public Health Service.

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