BINDING OF 2,3-DIPHOSPHOGLYCERATE (2,3 DPGA) TO HEMOGLOBIN
Isolation of peptides containing firmly bound 2,3 DPGA

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1. Introduction

There is presently much interest [1] in 2,3 diphosphoglycerate as a modifier of hemoglobin conformation; it is of interest, therefore, to clarify binding of 2,3 DPGA to hemoglobin. "Stripped" hemoglobin described by Benesch and Benesch [1], and claimed by these workers to be free from 2,3 DPGA, has shown consistently in this laboratory over 2 mμmoles 2,3 DPGA per mg hemoglobin. Moreover, 2,3 DPGA binds extensively to oxyhemoglobin [2]; this is also in contradiction to work of Benesch and Benesch [1].

2. Materials

All experiments were carried out with human oxyhemoglobin (red cells were washed 3 times with 0.15 M NaCl, lysed by freezing in distilled H_2O and then centrifugated at 20,000 X *g*). Radioactive 2,3-diphosphoglycerate ($D^{32}PGA$) was prepared from heparinized blood as previously described [3]. Oxyhemoglobin containing bound $D^{32}PGA$ was obtained from an aliquot of this mixture.

3. Results

Hemoglobin passed through Sephadex G-25 fine columns retains 2,3 DPGA, the amount depending upon the column dimensions. For example, when 2 ml of hemoglobin were passed through 2.5 X 30 cm columns as described by Benesch et al. [1], the hemoglobin retained 2–3 mμmoles of 2,3 DPGA per mg

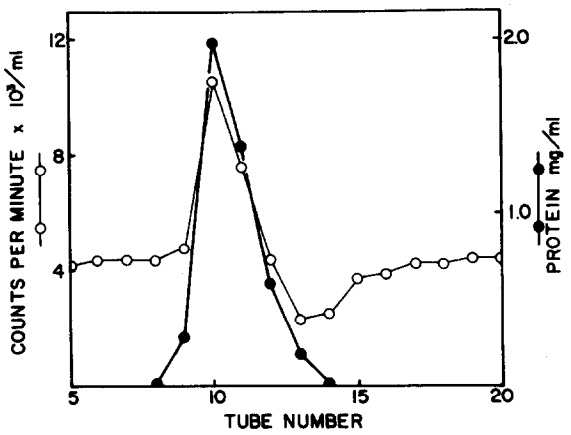

Fig. 1. Binding of $D^{32}PGA$ by oxyhemoglobin. A mixture containing 0.085 mM oxyhemoglobin and 0.012 mM $D^{32}PGA$ at pH 7.0 in 0.05 M NaCl was let stand for 1 hr at 24°. One-half ml portions were passed through a 0.5 X 110 cm column of Sephadex G-25 fine, equilibrated with 0.012 mM $D^{32}PGA$-0.05 M NaCl, flowing at 10 ml/hr. One ml aliquots were collected and assayed for hemoglobin and radioactivity.

(molar ratio 2,3 DPGA/hemoglobin 0.12 to 0.15). However, using 1.2 X 100 cm columns (20 ml/hr flow rate), the molar binding ratios decreased to 0.04 to 0.06. Longer columns will not remove additional 2,3 DPGA since it is firmly bound (see below).

Fig. 1 illustrates that oxyhemoglobin indeed binds one mole of 2,3 DPGA/mole. Nevertheless, as indicated, there is residual 2,3 DPGA bound to oxyhemoglobin which cannot be removed by gel filtration. Fig. 2 illustrates the incompleteness of $D^{32}PGA$ re-

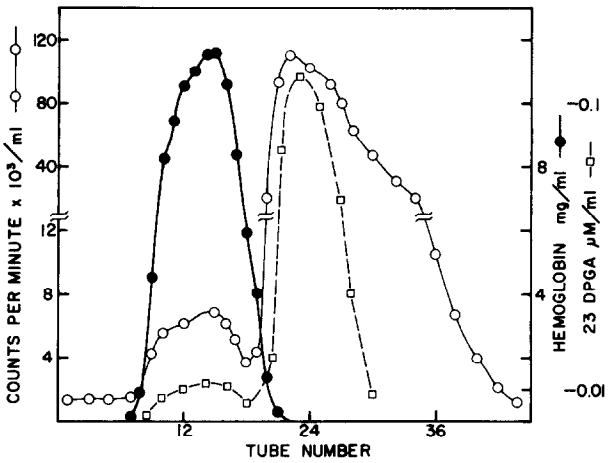

Fig. 2. Firmly bound $D^{32}PGA$ content of oxyhemoglobin. One and one-half ml samples of a solution containing 1.2 mM oxyhemoglobin and 1.2 mM $D^{32}PGA$ were passed through a 1.2 X 95 cm column of Sephadex G-25 fine, flowing at 20 ml/hr, equilibrated with 0.1 M NaCl at 24°C. Aliquots of 1.5 ml were collected and analyzed for hemoglobin, 2,3 DPGA and radioactivity. Aliquots were counted in a liquid scintillation counter using the systems described by Patterson and Greene [4].

moval from hemoglobin by Sephadex gel filtration. The molar binding ratio for residual bound 2,3 DPGA/hemoglobin is 0.067 and both radioactivity and enzymatic measurements agree very well. The reason that we are able to detect that ca. 10% of the average 2,3 DPGA present in the red cell [5] remains bound to hemoglobin after passage through Sephadex, in disagreement with others [1], is attributable to the use of a highly sensitive and specific enzymatic assay [5].

There are strong similarities between the binding of 2,3 DPGA to hemoglobin and the extensively studied binding of this reagent to 2,3 DPGA-dependent phosphoglyceromutase [6,7]. We tested whether 2,3 DPGA could be removed or converted to monophosphoglycerates on denaturation of the protein with acid or heat, as is the case with muscle phosphoglyceromutase [8]. Table 1 demonstrates that neither radioactivity nor 2,3 DPGA are released on denaturation of the protein. Moreover, 2 ml samples of the hemoglobin preparation were percolated through a 1 X 10 cm column of Dowex 1 X 8 resin, Cl^- form. The resin was washed with water and then with 0.5 N HCl-0.5 M NaCl. The protein, which was not adsorbed by the resin, retained all of the radioactivity and 2,3 DPGA. This treatment dissociates bound 2,3 DPGA from phosphoglyceromutase [8].

To further clarify the binding, separation of tryptic peptides was carried out. A 2 ml mixture of 0.2 mM oxyhemoglobin, 0.2 mM $D^{32}PGA$ and 0.05 M NaCl was incubated at 37° for 1 hr. Then, 0.1 ml of 1 M Na_2HPO_4 was added and the mixture was heated in boiling water for 4 min. After cooling, 1 mg of trypsin was added and the suspension incubated for 3 hr at 38°. After acidification to pH 6.4, soluble peptides were separated as illustrated in fig. 3. A 0.5 ml aliquot of the soluble peptides was passed through a 0.5 X 110 cm column of Sephadex G-10 equilibrated with 0.2 mM $D^{32}PGA$ in 0.05 M NaCl. Fig. 4 illustrates the peptide and radioactivity elution profiles and demonstrates binding of $D^{32}PGA$ to peptide(s). Aliquots of the tube containing the peak radioactivity were examined by thin layer (see fig. 3), and compared with the original digest. This peak contained

Table 1
Effects of heat and acid denaturation on residual 2,3 DPGA bound to oxyhemoglobin.

Experimental conditions	Protein mg/ml	P^{32}cpm/ml	2,3 DPGA mM
Control	8.45	4,500	0.009
95 supernatant	0.4	500	<0.001
$HClO_4$	0.2	240	<0.001

One ml aliquots of hemoglobin passed through Sephadex were heated in a boiling water bath for 4 min. The tubes were centrifuged and the supernatants fluids tested for protein, 2,3 DPGA and radioactivity. One ml of 6% $HClO_4$ was added to 1 ml samples. The supernatant fractions after centrifugation were adjusted to pH 7 with NaOH and assayed.

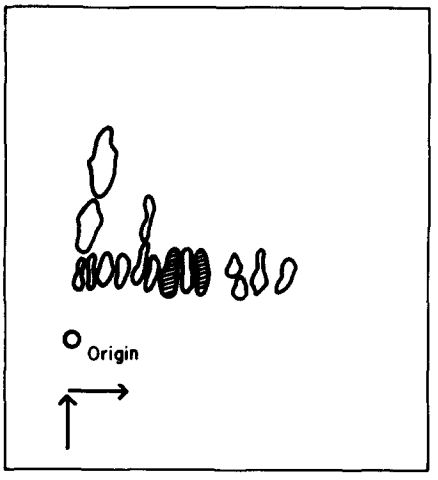

Fig. 3. Separation of tryptic peptides on cellulose thin layer plates by electrophoresis in pyridine-acetate buffer, pH 6.5, at 250 V for 2 hr followed by chromatography in *n*-butanol-pyridine-acetic acid-water (45:36:9:36). Peptides, detected with ninhydrin spray, are illustrated.

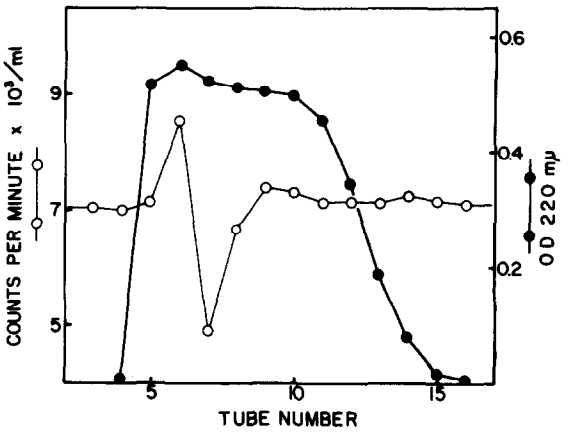

Fig. 4. Binding of $D^{32}PGA$ by tryptic peptides. Details are given in the text.

two major peptides, denoted as crosshatched spots in fig. 3.

4. Discussion

As tested by both radioactivity and by enzymatic assay, the amount of firmly bound 2,3 DPGA is small, ca 5% of the total 2,3 DPGA *which may be bound to oxyhemoglobin* (see fig. 1). The binding may be similar to muscle phosphoglyceromutase binding of 2,3 DPGA; this enzyme is not isolated as a phosphoenzyme nor is there detectable bound 2,3 DPGA. However, even if the amount of P-enzyme or bound 2,3 DPGA is very small, it is sufficient to maintain catalytic activity [9]. This behavior is even more extreme in the case of the yeast enzyme [10]. Nevertheless, the basic mechanism for both enzymes most likely involves covalent interaction of the cofactor and the protein. Alternatively, and since only ca 1/20 mole of 2,3 DPGA per mole of hemoglobin is bound, a certain type of hemoglobin may bind the material. Possibly either newly formed or older hemoglobin may bind the 2,3 DPGA. It is of much interest that red cells possess a small amount of adenoyl 2,3 DPGA (ca. 5% the amount of 2,3 DPGA) [11].

Experiments to further clarify the above points, particularly the identity of the peptide containing firmly bound 2,3 DPGA, and to compare the behavior of abnormal hemoglobins are in progress.

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