*THE INTERACTION OF HEMOGLOBIN AND ITS SUBUNITS WITH 2,3-DIPHOSPHOGLYCERATE**,†*

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We have recently shown that while the oxygen affinity of purified hemoglobin is very much greater than that of whole blood, the difference can be accounted for by the influence of red cell organic phosphates acting as cofactors (Benesch and Benesch, 1967; Benesch, Benesch, and Yu, 1968). Since D-2,3-diphosphoglycerate (DPG) accounts for the major portion of the phosphates in the human erythrocyte (Bishop, 1964), our studies were carried out with this compound, although some other phosphate esters, such as adenosine 5′-triphosphate (ATP), have similar effects (Benesch and Benesch, 1967).

The dramatic shift of the oxygenation curve of hemoglobin toward lower oxygen affinities in the presence of DPG is caused by a reciprocal affinity of hemoglobin for DPG and oxygen. Therefore, the addition of DPG to oxygenated hemoglobin liberates oxygen and oxygenation of deoxygenated hemoglobin displaces bound DPG. This is due to the fact that DPG is bound to deoxyhemoglobin, but not to oxyhemoglobin, under physiological conditions, in a ratio of one mole of DPG per mole of hemoglobin tetramer (Benesch *et al.*, 1968). The energy of binding accounts satisfactorily for the stabilization of the deoxy form of the protein by DPG and the consequent shift in the oxygenation curves. It is pertinent that the ratio of the molar concentration of effective organic phosphates in the human erythrocyte to that of hemoglobin is between 1.0 and 1.5 under normal conditions (Guest, 1942).

We have now investigated the interaction of DPG with the isolated α and β chains of human hemoglobin and correlated the results with the effect of DPG on the oxygenation behavior of these subunits.

Experimental.—Preparation of hemoglobin A and its subunits: Hemoglobin was prepared from freshly drawn human blood at weekly intervals as described previously (Benesch *et al.*, 1968). The α and β chains were separated by the method of Tyuma, Benesch, and Benesch (1966) based on that of Bucci and Fronticelli (1965).

After demercuration on thiolated Sephadex, the α chains were freed of all phosphate by rapid dialysis as described by Englander and Crowe (1965) against six changes of 500 ml 0.1 *M* NaCl (total dialysis time 3 hr at room temperature).

For the removal of mercury from the β chains, a procedure that is more suitable for preparing larger amounts was developed; 1 *M* *N*-acetyl-DL-penicillamine (Aldrich Chemical Co., Milwaukee, Wis.) was added to a 2% solution of the mercurated protein in 0.1 *M* phosphate buffer, pH 7.5, to give a final concentration of 0.1 *M*. After 1 hr at 4°C, the solution was dialyzed through two-dimensionally stretched Visking sausage casing (Craig, 1965) in the Englander apparatus against 100 vol of 0.1 *M* phosphate buffer, pH 7.5, for 1 hr at 4°C. The treatment with *N*-acetyl-penicillamine followed by dialysis was repeated twice more, and finally the protein was subjected to continuous dialysis against 8–10 liters 0.1 *M* NaCl at the rate of 500 ml/hr. The resulting preparation was free of phosphate and methemoglobin and contained not more than 0.01 atom of mercury/chain. *N*-acetyl-penicillamine, in contrast to many other thiols, does not lead to methemoglobin formation in the presence of oxygen. At the same time it is effi-

cient in removing mercury from proteins and has been used for this purpose *in vivo* (Aposhian, 1960).

Oxygen dissociation curves were determined as described earlier (Benesch, Macduff, and Benesch, 1965).

DPG (Calbiochem) was converted to the free acid with Dowex 50 and then used for binding experiments by the ultrafiltration method previously described (Benesch *et al.*, 1968).

All the binding experiments were performed at room temperature, pH 7.3, in 0.1 *M* NaCl. The method of Ames and Dubin (1960) was used to determine total phosphate in the ultrafiltrates.

The mercury content of the various protein fractions was determined by the method described in a previous paper (Tyuma *et al.*, 1966).

Results and Discussion.—Experiments on the binding of DPG to α and β subunits revealed that, while the α chains do not react with DPG in either the oxy or deoxy form (Table 1), both forms bind it equally in the case of the β chains (Fig. 1). Since the organic phosphate therefore shows no *preferential* affinity for the oxy or deoxy form of either subunit, it should be without effect on the oxygen affinity of these hemoproteins. The results shown in Figure 2 indicate that this is indeed the case.

It is, of course, very significant that only the β chains bind DPG since, like hemoglobin A, they exist as tetramers (β_4^A) (Benesch, Ranney, Benesch, and Smith, 1961), while the α chains do not (Huehns, Shooter, Dance, Beaven, and Shooter, 1961). On the other hand, mercurated β chains that have lost the ability to form tetramers (Bucci *et al.*, 1965) bind only minimal amounts of DPG. The binding of a maximum of one mole of DPG per β_4 tetramer is therefore analogous to that previously found with deoxyhemoglobin A. (The affinity of DPH for β_4^A is somewhat lower than that for deoxyhemoglobin A (K diss $= 7 \times 10^{-5}$ as compared with 2×10^{-5} for deoxyhemoglobin A) which may be connected with the lower isoelectric point of β_4^A.)

The finding that β_4^A binds DPG to the same extent in the oxy and deoxy form is in perfect accord with the well-established fact that, unlike hemoglobin A, β_4^A does not undergo a measurable conformational change with oxygenation (Benesch and Benesch, 1964; Perutz and Mazzarella, 1963). It is even more significant that the β_4^A tetramer behaves like the *deoxy* form of hemoglobin A in binding DPG, since Perutz and Mazzarella (1963) concluded that the structure of β_4^A both in the presence and absence of oxygen closely resembles that of deoxyhemoglobin A rather than oxyhemoglobin A. The similarity of β_4^A to deoxyhemoglobin A is also borne out by its resistance to splitting by concentrated sodium chloride (Benesch, Benesch, and Macduff, 1964).

As was pointed out by these authors, however, a deoxy conformation has been

TABLE 1. *Recovery of DPG from α chains.*

Concentration of α chains (gm/100 ml)	Concentration of DPG ($M \times 10^5$) Added	Found in ultrafiltrate
Oxy 0.26	5.4	5.6
Oxy 0.43	11.5	11.4
Deoxy 0.21	11.2	11.3
Deoxy 0.26	5.4	5.3
Deoxy 0.43	11.5	12.1

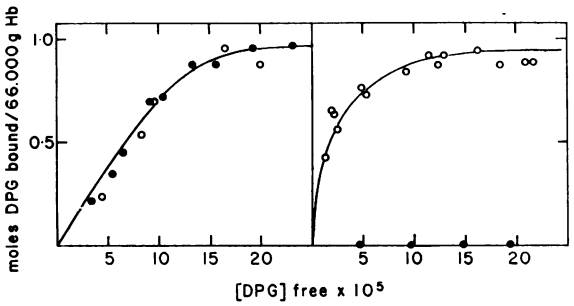

FIG. 1.—DPG binding by hemoglobin A and isolated β chains. All measurements in 0.1 *M* NaCl, pH 7.3, at 22° ± 2°C.
(*Left*) β_4^A; (*right*) hemoglobin A.
Open circles, deoxyhemoglobin; *filled circles*, oxyhemoglobin.

difficult to reconcile with the high oxygen affinity of this protein, which is in the range of that of myoglobin. The apparent paradox can now be resolved by the realization that the oxygen affinity of *phosphate-free* hemoglobin A is actually also in the same range as that of β_4^A or myoglobin (Figs. 2 and 3).

The shift toward lower oxygen affinity of hemoglobin A in the presence of DPG arises from the fact that the oxygen affinity is diminished by the energy required to dissociate the deoxyhemoglobin-DPG complex (6.400 cal/mole). β_4^A, on the other hand, can bind oxygen and DPG simultaneously and therefore its oxygen affinity remains high even in the presence of DPG.

The discovery that the oxygen affinity of "stripped" hemoglobin is of such a high order led us also to re-evaluate the relative affinities of hemoglobin and myoglobin for oxygen. In contrast to the widely accepted view that the oxygen affinity of hemoglobin is much lower than that of myoglobin, the curves in Figure 3 show that this is not the case when "stripped" hemoglobin is compared with myoglobin. It can be seen that over the last 25 per cent of the oxygenation reaction the two curves actually coincide. It must therefore be concluded that the intrinsic oxygen affinities of a single unoxygenated heme in hemoglobin and the only heme of myoglobin are very similar. (This bears out the correctness of Wyman's assumption that cooperative oxygen binding in hemoglobin is due to stabilizing interactions in deoxy rather than in oxyhemoglobin (Wyman, 1948).)

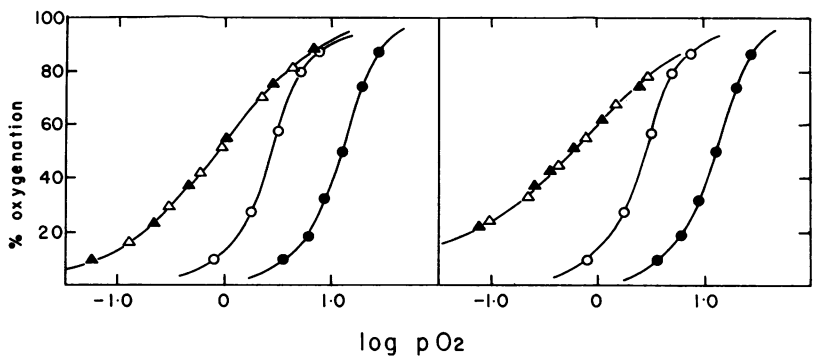

FIG. 2.—Effect of DPG on the oxygen equilibrium curves of hemoglobin A and its subunits. Hemoglobin concentration 0.3%, pH 7.0 (before deoxygenation), temperature 30°C.
(*Left*)—*Triangles*, α chains; *circles*, hemoglobin A.
(*Right*)—*Triangles*, β chains; *circles*, hemoglobin A.
Open symbols, in 0.01 *M* NaCl; *filled symbols*, in 0.01 *M* NaCl and 1×10^{-4} *M* DPG.

Since it is now established that in both proteins the same kind of heme is attached by the same bond to a polypeptide chain of very similar conformation (Cullis, Muirhead, Perutz, Rossmann, and North, 1962; Kendrew, Watson, Strandberg, Dickerson, Phillips, and Shore, 1961), this finding makes eminent sense. In the presence of physiological concentrations of DPG, on the other hand, the oxygen dissociation curve of hemoglobin is shifted to that of whole blood (Fig. 3).

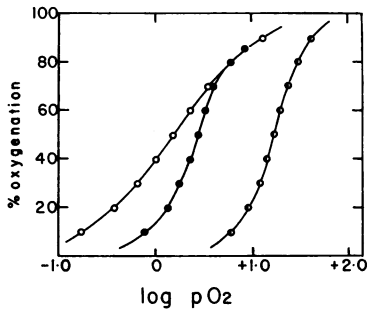

FIG. 3.—Comparison of the oxygenation curves of myoglobin, "stripped" hemoglobin, and whole blood.

Open circles, myoglobin at 30° plotted from the data of Rossi-Fanelli and Antonini (1958).

Filled circles, 0.3% "stripped" hemoglobin in 0.01 *M* NaCl at 30°, pH 7.0 (before deoxygenation).

Half-filled circles, whole blood at 30°C plotted from the data of Astrup, Engel, Severinghaus, and Munson (1965).

Since DPG reacts with hemoglobin in a mole-for-mole ratio, it must be assumed that it is bound on the diad axis of symmetry. The central cavity which is located along this axis (Bragg and Perutz, 1954; Cullis, Muirhead, Perutz, Rossmann, and North, 1962; Perutz, 1965) seems the most probable site, since its width changes with oxygenation. The entrance to this cavity is too small to admit DPG in the oxyhemoglobin molecule, but it becomes much wider in the deoxy form (Muirhead, Cox, Mazzarella, and Perutz, 1967; Bolton, Cox, and Perutz, 1968). This would account for the finding that DPG is not bound by oxyhemoglobin A, but combines with deoxyhemoglobin A and with both forms of β_4^A. The failure of α chains or of mercurated β chains to bind DPG must, of course, be viewed in the light of their inability to form tetramers.

Summary.—Under physiological conditions, i.e. pH 7.3, in 0.1 *M* NaCl, human hemoglobin binds 2,3-diphosphoglycerate only in the deoxy form in a ratio of one mole per hemoglobin tetramer. By contrast, hemoglobin H, the β_4^A tetramer, binds DPG equally in the oxygenated and deoxygenated state and in the same ratio as deoxy hemoglobin A. Isolated α chains, on the other hand, which are not tetrameric, do not react with DPG at all. The implication of these results for the site and the mechanism of binding of DPG to hemoglobin, as well as for the conformational changes accompanying oxygenation in these hemoproteins were discussed.

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