Organic phosphates increase the solubility of avian haemoglobin D and embryonic chicken haemoglobin

Rosemarie BAUMANN,* Eberhard GOLDBACH,* Ernst-A. HALLER* and Peter G. WRIGHT†

*Zentrum Physiologie, Medizinische Hochschule Hannover, Konstanty-Gutschow-Strasse 8, D-3000 Hannover 61, Federal Republic of Germany, and †Department of General Physiology, Faculty of Dentistry, University of Witwatersrand, Johannesburg, South Africa

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The isolated minor haemoglobin fractions (haemoglobin D) of ostrich, chicken and duck haemoglobin, which constitute about 30% of total intracellular haemoglobin, form crystalline aggregates upon deoxygenation at physiological temperature, ionic strength and pH and at haemoglobin concentrations even well below those present in the red cell. The aggregation is reversed by oxygenation, and can be inhibited by addition of organic phosphates or the corresponding major haemoglobin fraction in a stoichiometric ratio of 1:1. Embryonic haemoglobin from chicken has similar characteristics with respect to its solubility. The results indicate close functional homology of α^D and embryonic π -chains as well as a novel role for organic phosphates in the regulation of haemoglobin function.

The physiological function of haemoglobin requires an unusually high solubility. In mammalian and avian red cells the haemoglobin concentration is generally about 7–8 mmol of Hb₄ (tetramer)/kg of cell water, and it has been pointed out by Riggs (1976) that solubility must have imposed considerable evolutionary constraints. Several mammalian and non-mammalian haemoglobins readily form crystalline aggregates (cf. Riggs, 1976) once they are removed from the intact red cell to slightly less physiological conditions, and it is reasonable to assume that intracellular solubilization of a particular haemoglobin may in some cases only be achieved through interaction with other cellular components. This question is especially pertinent with regard to the multiple haemoglobins found in many species, since, as shown for HbS, interaction between haemoglobins can increase their solubility (Benesch *et al.*, 1980).

Compared with other vertebrate classes, the multiple haemoglobin pattern of avian species is remarkably stable. In most cases one observes two fractions, HbA ($\alpha_2^A\beta_2$) and HbD ($\alpha_2^D\beta_2$) in the nomenclature of Brown & Ingram (1974), where the minor fraction HbD accounts for between 15 and 30% of the total Hb (Saha & Ghosh, 1965).

Morrow *et al.* (1974) observed that HbD from

chicken crystallized readily upon deoxygenation at room temperature, and the aggregates dissolved when the protein was liganded.

In view of the widespread distribution of HbD among avian species, we have compared the solubility of three HbD components from chicken, ostrich and duck, as well as of embryonic-chicken haemoglobin, under physiological conditions of temperature, pH and ionic strength. The results show that in all cases the solubility of purified HbD or embryonic Hb in the deoxy form is well below the physiological haemoglobin concentration.

It was found that organic phosphates specifically increase the solubility of deoxy HbD or embryonic haemoglobin.

These results indicate a novel role of organic phosphates with respect to the regulation of haemoglobin function in avian blood, and the similarity of the behaviour of HbD and embryonic Hb also supports the notion that the avian α^D haemoglobin chain and the embryonic π -chain are much closer related than are the α^D - and α^A -chain (Czelusniak *et al.*, 1982).

Materials and methods

Haemoglobins A and D from ostrich, duck and White-Leghorn chicken were prepared by chromatography on a column (2.5 cm × 20 cm) of CM-cellulose CM52, a linear pH and salt gradient

Abbreviations used: HbA etc., haemoglobin A etc.; InsP₅, inositol pentakisphosphate; InsP₆, inositol hexakisphosphate.

(0.02M-phosphate buffer, pH 6.5; 0.05M-phosphate buffer, pH 8.0) being used. Organic and inorganic phosphates were removed from haemoglobin by two passages over a column (1.5 cm $\times$ 90 cm) of Sephadex G-25 (fine grade), equilibrated with 0.05M-Tris buffer (pH 8.8)/0.3M-NaCl. Throughout the preparation the haemoglobins were kept as carboxy Hb. Embryonic chicken haemoglobin was prepared from the blood of 6-day-old chicken embryos; adult human haemoglobin was obtained from blood donated (with consent) by members of the department. Phosphate-free haemoglobin solutions were dialysed against 100mM-NaCl and concentrated in an Amicon UF cell or with the Amicon Diaflo system. Samples were stored in liquid N₂ until use.

Isoelectric focusing was carried out on LKB Ampholines pH 7–9 as described by Drysdale *et al.* (1971), and estimates of the pI obtained by measuring the pH (at 20°C) in the gel fractions that had been cut and left overnight in 0.3 ml of 10mM-NaCl. Quantification of the gels was done by densitometry on a Gelman AC D15 densitometer. Haemoglobin concentration was determined after conversion into cyanmethaemoglobin, a ϵ_{546}^{mM} of 11.0 being used; CO was removed from haemoglobin by photolysis.

For determination of solubility, 100 μ l samples were deoxygenated by tonometry for 30 min in the Radiometer blood microsystem BMS 2 at 20 or 37°C and subsequently centrifuged for 2 min at 9600g under N₂ in a thermostatically controlled Eppendorf centrifuge. After centrifugation the haemoglobin concentration of the clear super-

natant was determined and taken as a measure of haemoglobin solubility. The pH of the samples was adjusted with NaOH or HCl. InsP₆ was purchased as the sodium salt from Fluka, and ATP from Boehringer.

Results and discussion

Solubility of HbD: effect of organic phosphates and CO₂

Differences in the solubility of deoxy- and oxy-haemoglobin were recognized very early (Jope & O'Brien, 1949), but the causes for this difference are still not understood. Generally it has been found for many globular proteins that, at moderate ionic strength, the solubility is lowest at the isoelectric point, due to the effect of the protein charge on the activity coefficient (cf. Tanford, 1961). The estimated isoelectric points for HbD from chicken, ostrich and duck are in the pH range between 7.7 and 7.9 at 20°C, and the temperature- and pH-dependence of the solubility of deoxy HbD (Figs. 1a–1c) conform with the abovementioned concept. Note that under our experimental conditions (0.1M-NaCl, 37°C and [total Hb] = 40g/litre) the solubility for all three HbD components in their deoxy form is far below the physiological requirements. At pH $\sim$ 7.2 the haemoglobin concentration of the soluble phase was 18.4g/litre for chicken HbD, 26g/litre for ostrich HbD, and 38.2g/litre for duck HbD (Table 1).

The red-cell concentration of HbD for these species is about 100g/litre of red blood cells, since it accounts for about 25–30% of the total haemo-

Fig. 1. pH- and temperature-dependence of solubility of deoxy HbD from (a) ostrich, (b) chicken and (c) duck. The total haemoglobin concentration was 40g/litre and that of NaCl, 0.1M.

Table 1. Concentration of deoxy HbD in supernatant at pH 7.2

Supernatant was obtained after centrifugation of the deoxygenated sample (2 min at 9600g). The total haemoglobin concentration was 40 g/litre and the solution contained 0.1 M-NaCl.

Species	Temperature (°C) . . .	[Deoxy HbD] (g/litre)	
		37	20
Chicken		18.4	13.2
Ostrich		26	22.4
Duck		38.2	33.4

Fig. 2. Influence of *InsP*₆ (a) and ATP (b) on the solubility of ostrich and chicken HbD
Conditions: temperature 37°C, [NaCl] 0.1 M, pH ~7.1.

globin. As intracellular crystallization of haemoglobin creates a serious hazard for red-cell stability, it is reasonable to assume that, *in vivo*, the solubility of deoxyhaemoglobin D has to be increased by other cellular constituents.

The most important allosteric effectors regulating avian haemoglobin function are *InsP*₅ for adult blood and ATP for embryonic and foetal blood (Bartlett & Borgese, 1976; Isaacks *et al.*, 1976). We therefore tested the effect of ATP and the *InsP*₅ analogue *InsP*₆ on the solubility of deoxy HbD. The results are shown in Figs. 2(a) and 2(b). It can be seen that both organic phosphates increase the solubility even when present at stoichiometric ratios of less than 1 mol of phosphate/mol of Hb₄. With a stoichiometric ratio of 1 mol of ATP (*InsP*₆)/mol of Hb₄, no crystallization was observed in the HbD concentration range 40–200 g/litre. A smaller but still significant increase of HbD solubility was also observed when the

Fig. 3. Influence of CO₂ (13.3 kPa) on the solubility of ostrich HbD
Conditions: temperature 37°C; total concentrations of Cl⁻ and HCO₃⁻ adjusted to 0.1 M.

samples were equilibrated with CO₂ (Fig. 3) at constant ionic strength of 0.1.

Two mechanisms may be responsible for the solubilizing effect of organic phosphates and CO₂: (i) the binding of these effectors changes haemoglobin net charge (Brygier *et al.*, 1975; Rossi-Bernardi & Roughton, 1967) and therefore the apparent pI; (ii) binding of organic phosphates, notably *InsP*₆ causes pronounced changes in the deoxyhaemoglobin structure (Arnone & Perutz, 1974), which may destroy intermolecular contact sites. Blocking of contact sites has been postulated as a mechanism for anti-sickling action of some chemical agents on human HbS (Noguchi & Schechter, 1978).

Recently it has been reported that *InsP*₆ can increase the solubility of liganded rat haemoglobin, an observation which was exclusively attributed to the transition from the R- to the T-state induced by the binding of *InsP*₆ (John, 1982).

Irrespective of the principal mechanism responsible for the solubilizing effect of organic phosphates and CO₂, the results point to an important new role of these effectors that has to be dissociated from that as regulators of haemoglobin oxygen affinity.

Comparison of solubility of HbD and embryonic-chick haemoglobin

The structural features causing the low solubility of pure HbD must be attributed to the α^D-chain, since HbA and HbD share a common β-chain (Brown & Ingram, 1974).

It has recently been proposed that the ancestral α^D -gene arose early in the evolution of haemoglobin (Czelusniak *et al.*, 1982), shortly before the gene duplication that produced the ancestral gene for embryonic α -like chains (i.e. the π -chain of chicken or the ζ -chain of human embryonic blood haemoglobin). This leads one to suspect a greater homology between the α^D and the embryonic α -like chains than between the α^D - and the α^A -chain.

Complete primary structures are available for the α^D -chain from chicken and pheasant (Takei *et al.*, 1975; Braunitzer & Godovac, 1982) as well as for the embryonic π -chain and the human ζ -chain (Chapman *et al.*, 1980; Aschauer *et al.*, 1981). All sequences show an unusual C-terminal section with glutamic acid at position $\alpha 138$, where one finds serine in human and most other mammalian haemoglobins and alanine in the α^A -chain from chicken haemoglobin (Dayhoff, 1975). Residues $\alpha 139$ to $\alpha 141$ play a very important role in subunit contacts (Fermi, 1975) and the introduction of a negatively charged residue at position $\alpha 138$ possibly leads to a considerable rearrangement of the C-terminus in deoxyhaemoglobin, which may have created a contact for intermolecular interaction.

Because of the limited amount of material available we could only carry out a few experiments testing the solubility of embryonic chicken deoxyhaemoglobin. As is shown in Table 2, embryonic chicken Hb behaves like HbD, especially with regard to the effect of organic phosphate on its solubility. These results support the close relationship between α^D - and the embryonic π -chains.

Effect of avian and human HbA on solubility of HbD

In the adult red cell or the emerging definitive red cell of embryonic blood, HbD is always in the presence of HbA. We therefore tested the effect of chicken and ostrich HbA on the solubility of HbD and found that in a 1:1 mixture of HbD and HbA in the concentration range from 40 to 200 g of total Hb/litre crystallization was inhibited completely.

Table 2. Solubility of deoxygenated haemoglobin solution from 6-day chicken embryo [70% HbP, 25% HbM + HbE, 5% adult Hb (HbA + HbD)]

Total haemoglobin concentration was 150 g/litre, except where marked *, where it was 40 g/litre. The temperature was 37°C. Note that the cell pH of 6-day embryonic cell is ~6.8 under physiological conditions (i.e. 37°C and pH_e 7.4).

pH	Condition	Fraction of soluble haemoglobin
6.945	0.1 mol of Cl ⁻	0.67
7.213	0.1 mol of Cl ⁻	0.91
6.850	1 mol of ATP/mol of Hb ₄	1.0
7.147	0.05 mol of Cl ⁻	0.46*

On the other hand, when human haemoglobin A was added to HbD from chicken under the same conditions, deoxy HbD crystallized extensively. The total haemoglobin concentration of the supernatant fell from 20 to 15.1 g/litre (mean for three experiments with chicken HbD), and isoelectric focusing showed that this was entirely due to loss of HbD from the soluble phase.

Thus the inhibitory effect of avian HbA on the aggregation of HbD evidently rests on specific interactions with HbD.

Conclusion

Avian HbD and embryonic haemoglobin from chicken possess an exceptionally low solubility when they are deoxygenated. Solubilization *in vivo* of the deoxy state is achieved through the joint effects of organic phosphates and the major avian haemoglobin, HbA.

The results indicate that the co-evolution of haemoglobin and organic phosphates as allosteric regulators may have been furthered by several causes aside from the need to adapt haemoglobin oxygen affinity. Embryonic haemoglobins have to rely entirely on organic phosphates for solubilization. In adult blood HbD can probably be sufficiently solubilized by HbA, which is present in excess of at least 2:1.

On the other hand, during foetal development it has been found that the first generations of definitive red cells contain more HbD than HbA (Bruns & Ingram, 1973), so that during this period organic phosphates are still important for solubilization. It is noticeable in this regard that, contrary to the ontogeny of mammals, where several species have low red-cell organic phosphate concentrations during late foetal development (cf. Jelkmann & Bauer, 1981), this has not been observed in any avian species so far. Instead the total red-cell concentration of organic phosphates in adult or embryonic/foetal blood is always kept at a ratio above 1 mol/mol of Hb, indicating a tightly regulated system, where maintenance of adequate solubility may have attributed important selective constraints.

References

- Arnone, A. & Perutz, M. F. (1974) *Nature (London)* **249**, 34–36
- Aschauer, H., Sanguansermisri, I. & Braunitzer, G. (1981) *Hoppe-Seyler's Z. Physiol. Chem.* **362**, 1159–1162
- Bartlett, G. R. & Borgese, I. A. (1976) *Comp. Biochem. Physiol.* **55A**, 207–210
- Benesch, R. E., Edalji, R., Benesch, R. & Kwong, S. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 5130–5134
- Braunitzer, G. & Godovac, J. (1982) *Hoppe-Seyler's Z. Physiol. Chem.* **363**, 229–238

- Brown, J. & Ingram, V. (1974) *J. Biol. Chem.* **249**, 3960–3972
- Bruns, G. A. P. & Ingram, V. M. (1973) *Philos. Trans. R. Soc. London* **266**, 225–305
- Brygier, J., De Bruin, S. H., Peter, M. K. B. & Rollema, H. S. (1975) *Eur. J. Biochem.* **60**, 379–383
- Chapman, B. S., Tobin, A. J. & Hood, L. E. (1980) *J. Biol. Chem.* **255**, 9051–9059
- Czelusniak, J. O., Goodman, M., Hewett-Emmett, D., Weiss, M. L., Venta, P. J. & Tashian, R. E. (1982) *Nature (London)* **298**, 297–300
- Dayhoff, M. O. (1975) *Atlas of Protein Sequence and Structure*, National Biomedical Research Foundation, Washington, DC
- Drysdale, J. W., Piergiorgio, R. & Bunn, H. F. (1971) *Biochim. Biophys. Acta* **229**, 42–50
- Fermi, G. (1975) *J. Mol. Biol.* **97**, 237–256
- Isaacks, R. E., Harkness, D. R., Froeman, G. A., Goldman, P. H., Adler, J. L., Sussmann, S. A. & Roth, S. (1976) *Comp. Biochem. Physiol.* **53A**, 151–156
- Jelkmann, W. & Bauer, C. (1981) *Acta Biol. Med. Germ.* **40**, 661–664
- John, M. J. (1982) *Eur. J. Biochem.* **124**, 305–310
- Joep, H. M. & O'Brien, J. R. P. (1949) in *Haemoglobin* (Roughton, F. J. W. & Kendrew, J. C., eds.), p. 269, Butterworths, London
- Morrow, J. S., Wittebrot, R. J. & Gurd, F. R. N. (1974) *Biochem. Biophys. Res. Commun.* **60**, 1058–1065
- Noguchi, C. I. & Schechter, A. N. (1978) *Biochemistry* **17**, 5455–5459
- Riggs, A. (1976) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **35**, 2115–2118
- Rossi-Bernardi, L. & Roughton, F. J. W. (1967) *J. Physiol. (London)* **189**, 1–29
- Saha, A. & Ghosh, J. (1965) *Comp. Biochem. Physiol.* **15**, 217–237
- Takei, H., Ota, Y., Wu, K., Kiyohara, I. & Matsuda, G. (1975) *J. Biochem. (Tokyo)* **77**, 1345–1347
- Tanford, C. (1961) *Physical Chemistry of Macromolecules*, pp. 240–242, John Wiley and Sons, New York