Pflügers Arch. 377, 75–80 (1978)

Pflügers Archiv
European Journal of Physiology

Embryonic Hemoglobins: Dependency of Functional Characteristics on Tetramer Composition*

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Abstract. 1. Nucleated erythroblasts from embryonic rabbits contain two groups of tetrameric hemoglobins (Hbs): Hbs $E_{I\text{-}III}$ consist of embryonic α-type chains (χ-chains) and embryonic β-type chains (ε-chains) whilst Hbs $L_{I\text{-}III}$ are composed of adult α-chains and ε-chains. Structural analyses have indicated that the χ-chains are evolutionarily older than ε-chains. To obtain informations on possible differences in ligand binding properties associated with these embryonic chains, we have prepared Hbs $E_{I\text{-}III}$ and $L_{I\text{-}III}$ from the erythroblasts of 14-days-old embryonic rabbits and measured their oxygen affinity at various pH values and different concentrations of phosphate compounds. These data were compared with those obtained on the unfractionated embryonic hemolysate and adult rabbit hemoglobin (HbA).

2. We found that Hbs $E_{I\text{-}III}$ have a higher oxygen affinity than Hbs $L_{I\text{-}III}$ at all pH values investigated, the difference becoming larger at more acid pH. As a result, the Bohr effect is smaller in Hbs $E_{I\text{-}III}$ than in Hbs $L_{I\text{-}III}$, $\Delta \log P_{50}/\Delta \mathrm{pH}$ amounting to −0.25 and −0.50, respectively. In the pH range between 6.8 and 7.8 the oxygen affinities of HbA and of Hbs $L_{I\text{-}III}$ are alike but lower in HbA at more acid pH. These results indicate that the presence of embryonic χ-chains in hemoglobin tetramers raise the oxygen affinity and lower the Bohr effect of the pigment, whereas the combination of adult α-chains with embryonic ε-chains lead to hemoglobin tetramers with a very similar oxygen affinity to HbA in the physiological pH range. The cooperativity of oxygen binding was smaller both in Hbs $E_{I\text{-}III}$ and $L_{I\text{-}III}$ compared to HbA.

3. The effect of added phosphates notably of 2,3-diphosphoglycerate (2,3-DPG) on the oxygen affinity of Hbs $E_{I\text{-}III}$ and $L_{I\text{-}III}$ was very similar, i.e. the rise in P_{50} produced by maximal concentrations of 2,3-DPG was not significantly different in the two types of embryonic hemoglobins. In HbA, the increase of P_{50} produced by comparable concentrations of 2,3-DPG was only slightly higher than in the embryonic hemoglobins. This shows that the embryonic ε-chains are similarly effective in binding phosphate as the adult β-chains.

Key words: Embryonic hemoglobins – Oxygen affinity – Bohr effect – Phosphate effect.

* Supported by the Deutsche Forschungsgemeinschaft

Introduction

Embryonic hemoglobins are the first respiratory pigments which transport oxygen in the circulatory system during the early stages of mammalian development. They are tetramers which contain specific embryonic α- or β-type chains [11, 16, 17, 20, 25]. The embryonic α-chains have been designated by the greek letters ζ (human) or χ (rabbit, mouse), and the embryonic β-chains by an ε to which a latin letter may be added to indicate that the ε-chain in question is different in amino acid composition from another ε-chain. Such, the human embryonic hemoglobin Portland ($\zeta_2\gamma_2$) consists of two identical α-type chains (ζ-chains) and the same two γ-chains which are found in human fetal hemoglobin [5, 8, 14, 15, 26, 28]. Two other human embryonic hemoglobins are named Gower I and Gower II. Gower I is thought to consist of ζ-chains and ε-chains [10] whilst Gower II has the structure $\alpha_2\varepsilon_2$, i.e. it contains adult α- and embryonic β(ε)-chains [10, 11, 25]. In the mouse three embryonic hemoglobins are present, one of which (E_1) consists of embryonic α- and embryonic β-chains ($\chi_2\varepsilon y_2$) whilst hemoglobin E_2 ($\alpha_2\varepsilon y_2$) and E_3 ($\alpha_2\varepsilon z_2$) are made up from adult α- and embryonic β-chains [18, 22, 23].

0031-6768/78/0377/0075/$1.20

The nucleated erythroblasts of embryonic rabbits contain six embryonic hemoglobin types which can be divided into two groups according to their tetrameric composition which results in a different eluting behaviour on carboxymethyl cellulose [23]. Hemoglobins E_{I-III} represent the early eluting group and consist of χ- and ε-chains whilst hemoglobins L_{I-III} which elute late, are composed of adult α- and ε-chains [22, 23]. Interestingly, the embryonic α-type chains from man, mouse and rabbit exhibit much more sequence similarity among each other than with the adult α-chain of the respective species. These structural characteristics indicate the embryonic α-chains have diverged from the main α-chain line well before mammals appeared in the course of vertebrate phylogeny [18, 23]. Embryonic ε-chains of mouse and rabbit on the other hand, show a pronounced sequence similarity with the corresponding adult β-chains suggesting a much younger evolutionary age [23].

The functional relationship between ligand binding properties and tetrameric assembly of embryonic hemoglobins, however, has not yet been established. The human embryonic hemoglobin Portland has a much higher oxygen affinity than either human fetal or adult hemoglobin if compared at pH 7.2 and 25° C [2, 27] and shows furthermore a significantly smaller alkaline Bohr effect than HbA [27]. Strikingly similar functional differences are observed between embryonic and adult mouse hemoglobins [3]. The evolutionary stability of the embryonic α-type chains as well as the structural similarity between embryonic ε- and adult β-chains suggests that the distinctive functional features of embryonic hemoglobins are mainly due to the presence of embryonic α-chains in the tetramer [23]. However, the experimental evidence obtained so far does not permit any definite conclusion in this respect. The present investigation was carried out on the embryonic hemoglobins E_{I-III} and L_{I-III} from the rabbit to obtain informations on the functional contribution of embryonic χ- and ε-chains.

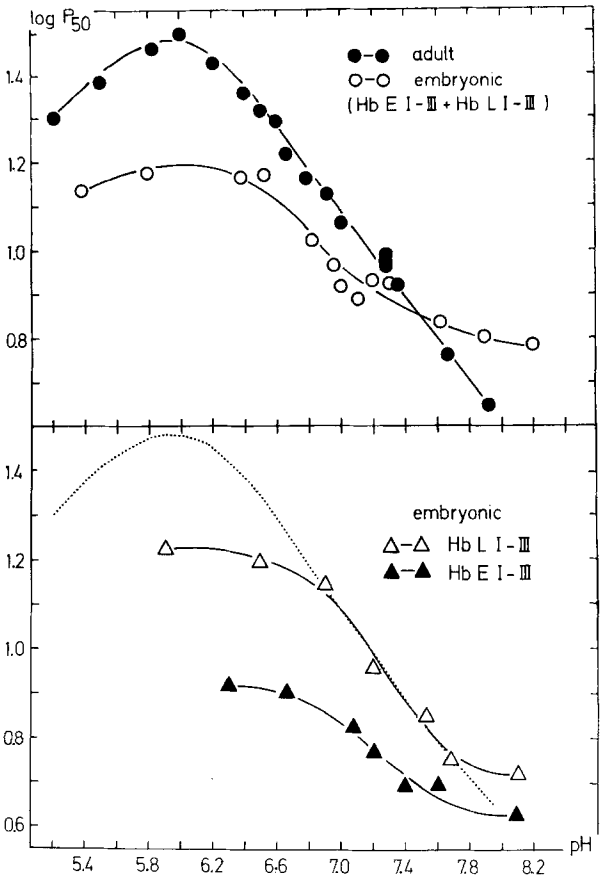

Fig. 1. *Upper Panel.* Plot of log P_{50} against pH for hemoglobins from adult (HbA) and embryonic rabbits. Embryonic hemoglobins (Hb E_{I-III} + Hb L_{I-III}) were not fractionated prior to oxygen binding experiments. *Lower Panel.* Plot of log P_{50} against pH for the chromatographically isolated embryonic hemoglobins E_{I-III} and L_{I-III}. Hemoglobins E_{I-III} consist of embryonic α- and embryonic β-type chains and hemoglobins L_{I-III} of adult α- and embryonic β-chains. The dotted line corresponds to the curve obtained for HbA. Embryos had a gestational age of 14 days. Temperature was 37°C, oxygen binding curves were done in the absence of CO_2. P_{50} is the oxygen pressure in Torr at which [Hb] = [HbO_2]. 1 Torr corresponds to 0.133 kPa

Materials and Methods

Nucleated erythroblasts were prepared from 14-days-old embryonic New Zealand white rabbits as previously described [13]. For the preparation of hemoglobin solutions, red cells were washed in saline, lysed by addition of distilled water and all phosphates removed from the hemoglobin either by gel filtration or on a mixed-bed ion-exchanger as described previously [12]. The hemoglobin samples to be subjected to ion exchange chromatography were gassed with carbon monoxide to prevent oxidation of the heme iron. Separation of hemoglobins E_{I-III} and L_{I-III} was carried out at 4° C on a 2 × 10 cm CM 52 (Whatman) column equilibrated with 0.01 mol · l^{-1} phosphate buffer, pH 6.4, containing 10^{-4} mol · l^{-1} EDTA. About 200 mg of embryonic hemoglobins was dialysed against the same buffer, the precipitate which formed during dialysis was removed by centrifugation and the clear supernatant charged on the column. The elution was carried out with 0.01 mol · l^{-1} Na_2HPO_4 containing 10^{-4} mol · l^{-1} EDTA at a flow rate of ~10 ml · cm^{-2} · h^{-1}. The gradient was adapted to this particular separation problem in pilot experiments and furnished by a variable gradient pump (Dialagrad Mod. 318; Isco Comp., Lincoln, Nebraska). The elution pattern of the various hemoglobin components resembled qualitatively that published by Steinheider et al. [22]. However, the use of an adapted gradient improved the chromatographic resolution in the present experiments. Fractions corresponding to either hemoglobins E_{I-III} or the hemoglobins L_{I-III} were pooled and concentrated with carbon monoxide using the Amicon system (PM-10 membranes in an UF cell, Amicon Corp., Lexington, Mass.). The concentrated material was then dialyzed at 4° C against 0.1 mol · l^{-1} NaCl. Subsequently, the CO was removed from the hemoglobin by exposing the pigment in a tonometer, cooled in an ice-water bath, to strong incandescent light under a stream of oxygen. Spectrophotometric analysis revealed that the hemoglobin contained less than 8% hemiglobin using the extinction coefficients

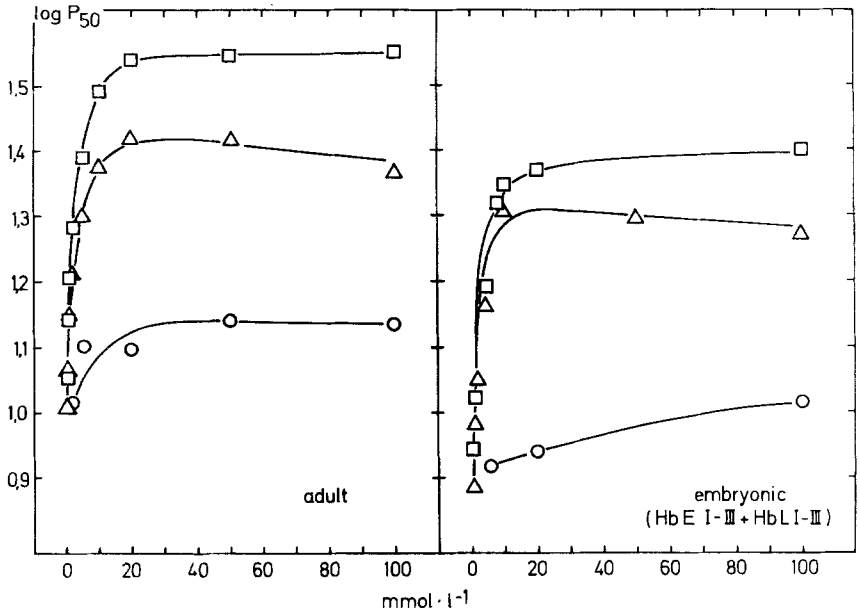

Fig. 2
Plot of log P_{50} against the concentration of 2,3-DPG (□——□), ATP (△——△) and inorganic phosphate (○——○) for adult HbA (left panel) and unfractionated embryonic hemoglobins $E_{I\text{-}III}$ + $L_{I\text{-}III}$ (right panel). Temperature was 37° C, oxygen binding curves were done in the absence of CO_2 at pH 7.2. P_{50} is the oxygen pressure in Torr at which [Hb] = [HbO_2]. 1 Torr corresponds to 0.133 kPa

published for human hemoglobin [1, 4]. The material was then stored in liquid nitrogen until needed.

Oxygen Binding Curves. Oxygen binding to hemoglobin was studied at 37° C at various pH values as well as in the absence and presence of added 2,3-diphosphoglycerate (DPG), adenosine triphosphate (ATP) and inorganic phosphate (P_i). Buffers were 100 mmol · l^{-1} bis-Tris at pH ≤ 7.5 and 100 mmol · l^{-1} Tris at pH > 7.5. Total chloride concentration was 150 mmol · l^{-1}. Oxygen binding curves were measured spectrophotometrically [19, 21] in an apparatus manufactured by Eschweiler Comp., Kiel. Rather than recording the whole oxygen binding curve, we have equilibrated the hemoglobin layer with 4 to 5 gas mixtures of O_2 and purified N_2 provided by gas mixing pumps (Wösthoff Comp., Bochum) covering the range between 15% and 85% saturation. Hemoglobin concentration was 2 mmol · l^{-1} Hb_4 for adult and unfractionated embryonic hemoglobins and 0.3 mmol · l^{-1} Hb_4 for hemoglobins $E_{I\text{-}III}$ and $L_{I\text{-}III}$. Changes in absorption were recorded at 576 nm and 436 nm for the more concentrated and dilute hemoglobin solutions, respectively. The monochromator of the apparatus has a half band width of ~4 nm at a slit width of 0.5 mm. In control experiments it was established that a 1:1 mixture of the solutions containing hemoglobins $E_{I\text{-}III}$ and $L_{I\text{-}III}$ yielded the same oxygen binding curves as the unfractionated hemolysate of embryonic hemoglobins.

Results and Discussion

In Fig. 1 are shown the curves relating log P_{50}, the partial pressure of oxygen at which the hemoglobin is half-saturated with oxygen, to pH. The range in which this relationship is linear can be used to calculate the Bohr coefficient $\Delta \log P_{50}/\Delta$ pH. It can be seen that at pH < 7.5 the adult rabbit hemoglobin has a lower oxygen affinity compared to the total unfractionated embryonic hemolysate and that this difference becomes larger as pH decreases (Fig. 1 upper panel). As a result, the Bohr coefficient in the alkaline range is higher in the adult ($\Delta \log P_{50}/\Delta$ pH = −0.50) than in the embryonic ($\Delta \log P_{50}/\Delta$ pH = −0.32) pigments. There is no clearcut dependency of the slope of the oxygen binding curves upon pH in embryonic and adult hemoglobins: the parameter n in the Hill equation [9] averaged 2.30 and 2.77, respectively. As will be seen later, the low degree of cooperativity in the embryonic hemoglobins cannot be exclusively explained on the basis of the functional heterogeneity of the various components of the hemolysate as both hemoglobins $L_{I\text{-}III}$ and hemoglobins $E_{I\text{-}III}$ which have different oxygen affinities, exhibit the same low n value as the whole hemolysate.

The decrease in oxygen affinity produced by phosphate compounds was similar in adult hemoglobin and the unfractionated embryonic hemolysate (Fig. 2). 2,3-DPG had the strongest effect, being followed by ATP and inorganic phosphate. Note that the ratio of P_{50} values in the absence and in the presence of saturating concentrations of 2,3-DPG was quite similar in the two types of hemoglobin suggesting that the ratio of the binding constants of 2,3-DPG to the oxygenated and the deoxygenated derivative is also similar in magnitude [24]. The slight fall in P_{50} at very high ATP concentrations seems to indicate that both in adult and embryonic hemoglobins, the oxygenated derivative has more than one binding site for ATP. Such an additional binding site in oxyhemoglobin decreases P_{50} at very high phosphate levels relative to the maximum effect produced at intermediate ATP concentrations [24]. A similar phenomenon was observed by Desbois and Banerjee [6] with human hemoglobin and the polyanion benzenehexacarboxylate but not with the polyanion inositolhexaphosphate.

Examination of the functional properties of hemoglobins $E_{I\text{-}III}$ which are composed exclusively of em-

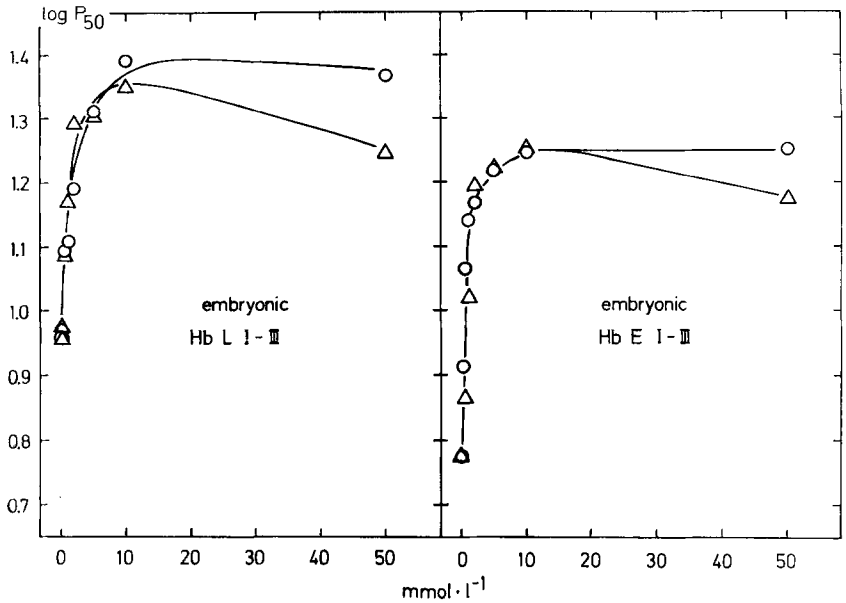

Fig. 3
Plot of log P_{50} against the concentration of 2,3-DPG (○——○) and ATP (△——△) for chromatographically isolated embryonic hemoglobins $L_{I\text{-}III}$ (left panel) and hemoglobins $E_{I\text{-}III}$ (right panel). Temperature was 37° C, oxygen binding curves were done in the absence of CO_2 at pH 7.2. P_{50} is the oxygen pressure in Torr at which [Hb] = [HbO_2]. 1 Torr corresponds to 0.133 kPa. For abbreviations of embryonic hemoglobins see legend to Fig. 1

bryonic globin chains [18,22] with those of hemoglobins $L_{I\text{-}III}$ which contain α- and embryonic ε-chains, clearly shows that hemoglobins $E_{I\text{-}III}$ have a higher oxygen affinity and a lower Bohr effect than hemoglobins $L_{I\text{-}III}$ (Fig. 1, lower panel). If one compares the relatively small linear range in the alkaline part of the Bohr effect curve it turns out that $\Delta \log P_{50}/\Delta$ pH is −0.50 in hemoglobins $L_{I\text{-}III}$, i.e. identical with adult hemoglobin, but only −0.25 in hemoglobins $E_{I\text{-}III}$. It thus becomes clear that, like in hemoglobin Portland, replacement of adult α-chains by embryonic α(χ)-chains leads not only to a reduction in the Bohr effect but also to a considerable increase in oxygen affinity at least in the physiological pH range. At pH 7.2, for example, P_{50} of hemoglobin Portland is about 2.5 times lower than of human fetal hemoglobin [2, 27] whilst hemoglobins $E_{I\text{-}III}$ have a 1.6 times lower P_{50} than hemoglobins $L_{I\text{-}III}$. Furthermore, the Bohr effect is halved both in hemoglobins $E_{I\text{-}III}$ and in hemoglobin Portland in comparison to the adult hemoglobin types [27].

As with the total hemolysate, there was no significant change in the slope of the oxygen binding curves with pH, Hill's n averaging 2.23 and 2.21 for hemoglobins $L_{I\text{-}III}$ and $E_{I\text{-}III}$, respectively. If the low degree of cooperativity was not a function of the presence of embryonic ε-chains in a tetramer, one would expect a further reduction of n in the total hemolysate as both groups of hemoglobins have different ligand affinities. At the extreme ends of the pH range investigated there seems to be a functional interdependence of the hemoglobins $E_{I\text{-}III}$ and $L_{I\text{-}III}$ which means that hybrid molecules are present which have a different oxygen affinity compared with the parent molecules. This conclusion is drawn from the observation that both at high and low pH values the oxygen affinity of the total hemolysate is somewhat lower than one would calculate from the P_{50} values of hemoglobins $E_{I\text{-}III}$ and $L_{I\text{-}III}$.

As far as the effect of organic phosphate compounds on the oxygen affinity of hemoglobins $E_{I\text{-}III}$ and $L_{I\text{-}III}$ is concerned, there was no significant difference in response to addition of organic phosphates (Fig. 3). In both groups of embryonic hemoglobins, the maximum effect was produced by 2,3-DPG. Again, the ratio of P_{50} of hemoglobins $E_{I\text{-}III}$ and $L_{I\text{-}III}$ in the absence of organic phosphates and at saturating concentrations of 2,3-DPG was quite similar (1.5 and 1.4, respectively) indicating that the embryonic ε-chains which most likely mediate the phosphate effect, are equally effective in binding phosphate esters as the adult β-chains and not influenced by the specific properties of embryonic χ-chains.

The question which then arises is related to the physiological significance of the specific properties of embryonic hemoglobins as a whole and of the differences between hemoglobins $E_{I\text{-}III}$ and $L_{I\text{-}III}$ in particular. All experimental studies obtained so far have shown that the intrinsic oxygen affinity of embryonic hemoglobins is higher than that of the adult pigment and that this difference is amplified in absolute terms by organic phosphate esters at least in embryonic hemoglobins from mice and rabbits. The same holds probably for the human embryonic hemoglobin Portland in which the presence of the fetal γ-chains should even reduce the effect of phosphates on the oxygen affinity. Nonetheless, measurements on the oxygen binding properties of nucleated erythroblasts from rabbits

which contain almost exclusively the embryonic hemoglobins E_{I-III} and L_{I-III} have shown that the oxygen affinity of these cells is just as low as that of erythrocytes from adult animals which led to the conclusion that the high concentration of organic phosphates and a relatively low pH in these erythroblasts largely determines their oxygen binding properties [13]. Similarly, human embryonic red cells which contain more than 30% of embryonic hemoglobins also have a lower oxygen affinity than one would expect from studies on the isolated pigments [10, 27]. These results demonstrate that the high oxygen affinity and low Bohr effect of the unfractionated embryonic hemoglobins are not directly reflected in the functional properties of red cells during the early phases of mammalian ontogeny.

The structural similarity of the embryonic α-type chains of man, mouse and rabbit [18] leads one to remember Haeckel's "biogenetic law" which states that certain features of phylogenetic development are recapitulated during the ontogeny of an individual. In terms of the evolution of hemoglobins, the "biogenetic law" implies that globin chains that function in an embryo are evolutionarily older than their adult counterparts. Zuckerkandl [29] in comparing the structure of various fetal γ-chains with adult α- and β-chains has come to the conclusion that "the theory of recapitulation seems to be altogether unsuccessful". The structural data on embryonic hemoglobin chains support his view as even among the earliest globin chains which are produced during mammalian development there are two groups of globin structures whose evolutionary history in terms of the date at which they arose by gene duplication is obviously very different: the embryonic α-type chains may have separated from the main α-chain line some 200–250 million years ago but the embryonic ε-chain resembles much more the more recent adult β-chain at least in mice and rabbits [23] and is therefore not expected to be much older than the respective species.

As mentioned earlier, the precise contribution of the various embryonic hemoglobins to the intrauterine development of mammals and therefore to mammalian phylogeny altogether is difficult to assess at present, mainly because of the lack of data on the respiratory gas exchange in early embryos and more extensive structural and functional studies on embryonic hemoglobins and red cells. It is hoped, however, that more experimental details on these respiratory pigments will eventually clarify the question if natural selection has guided the evolution not only of adult [7] but also of embryonic globin chains.

Acknowledgements. We thank Dr. H. S. Rollema for helpful discussions and Miss G. Wischka for the typing of the manuscript.

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Received April 12, 1978