Allosteric modulation of oxygen binding to the three human embryonic haemoglobins

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Plasmid based yeast expression systems have been developed for the high-level expression of the three human embryonic haemoglobins Gower I ($\zeta_2\epsilon_2$), Gower II ($\alpha_2\epsilon_2$) and Portland ($\zeta_2\gamma_2$). Physicochemical characterization of the three product haemoglobins show them to be in the 'native' state. Oxygen-binding studies show that, under what are usually considered physiological conditions, each of the embryonic haemoglobins shows a

high oxygen affinity, coupled to a high degree of co-operativity. Allosteric modulation of the oxygen-binding properties of the three haemoglobins in response to organic phosphates and protons has been investigated. The various responses exhibited by the three haemoglobins are rationalized in terms of their amino acid sequences.

INTRODUCTION

The development of the human individual can be divided into three temporal phases, namely post-natal, placental and pre-placental, or adult, fetal and embryonic. Each of these phases is associated with the synthesis of distinct classes of oxygen-transport molecules [1]. Adult haemoglobin (HbA; $\alpha_2\beta_2$) is produced in the bone marrow and is optimized for gas exchange at the lung surface. Fetal haemoglobin (HbF; $\alpha_2\gamma_2$) is produced predominantly in the liver during the placental phase and is optimized for oxygen transport across the placenta. In the embryonic phase three different haemoglobins are synthesized, namely haemoglobins Gower I ($\zeta_2\epsilon_2$), Gower II ($\alpha_2\epsilon_2$) and Portland ($\epsilon_2\gamma_2$) [2]. The embryonic haemoglobins produced during the pre-placental phase are themselves synthesized in a specific order. Haemoglobin Gower I is produced maximally between weeks 2 and 4 of gestation, and is followed by Portland and Gower II. All three embryonic haemoglobins are completely replaced in the circulation by fetal haemoglobin by week 14 of gestation. In the absence of detailed physiological information for the earliest stages of embryonic development, it is not possible to define the exact role of these haemoglobins, but clearly they play an important role in scavenging oxygen from the amniotic fluid before the formation of a fully functional placenta.

In the recent past the moral, ethical and practical problems associated with the study of molecules having their natural origins in human embryonic tissues have been circumvented by means of the application of recombinant technologies [3]. These technologies are themselves firmly based on earlier reports of the use of yeast expression systems in the production of adult haemoglobins [4–6]. This work has now made possible the study of the structure and function of this important class of oxygen-transport molecules.

Here we describe the use of a previously reported [3] plasmid-based yeast expression system capable of providing large quantities of each of the embryonic haemoglobins. The three embryonic haemoglobins have been purified and their oxygen-binding properties studied.

Furthermore, the response of each of the embryonic haemoglobins to changes in pH and the concentration of organic phosphates has been monitored. The disparate responses of the

three human embryonic haemoglobins to these different solution conditions can be rationalized in terms of the known amino acid sequences of each of the globin proteins.

MATERIALS AND METHODS

Plasmids suitable for the expression of the three human embryonic haemoglobins were created by using the procedures previously described [3]. Yeast cells were grown as described previously [7], with the exception that at the time of induction a further portion of ethanol (1%) was added to the culture to decrease the metabolic turnover of the galactose employed as the promoter. Protein purification, globin-chain separation, h.p.l.c. amino acid sequencing and molecular-mass determination were performed as previously described [3].

Oxygen-binding curves were obtained by using a HEMOX oxygen-dissociation analyser as described previously [3,5].

The allosteric responses of the embryonic haemoglobins were investigated in two different sets of experiments. In the first set of experiments oxygen-binding curves were measured in either 50 mM Tris buffer at pH values above 7.0 or 50 mM Bistris buffer at pH values below 7.0 in the presence of 100 mM NaCl. In this case two or three curves were measured in both buffers at identical pH values around neutrality to highlight any buffer-specific effects; within experimental error, none were detected. The Bohr coefficient was determined from the slope of the linear section of the graph of $\log p_{50}$ (oxygen partial pressure giving 50% saturation) versus pH obtained by linear least-squares fitting.

The effect of organic phosphates was measured by recording oxygen-binding curves at pH 7.4 in 50 mM Tris buffer in the presence of 100 mM NaCl supplemented with 2,3-bisphosphoglycerate in the range 0–5 mM. All p_{50} and Hill-coefficient (h) values presented here were determined from the experimental oxygen-binding curves by fitting the experimental data points to a sigmoidal function using non-linear least-squares fitting routines.

Haem:globin ratios were determined by integration of the h.p.l.c. peak of haem and globin protein for each chain, adult haemoglobin being used as an internal standard. The nature of the N-terminal blocking group of the ζ chain was determined by fast-atom-bombardment m.s. of the tryptic digest of the γ chain.

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RESULTS

Haemoglobin production and physicochemical characterization

When each of the embryonic haemoglobin expression systems in yeast was promoted by the addition of galactose, under previously described conditions [3], 70 mg of haemoglobin per litre of culture was produced after 16 h further culture. This haemoglobin was free of any sulphaemoglobin [7].

Samples of each haemoglobin, after the published purification procedure [3], were then subjected to molecular-mass determinations. SDS/PAGE showed each sample to be essentially pure and composed exclusively of protein chains of molecular mass 15 kDa. Each of the embryonic proteins, when in the carbonmonoxy form, exhibited an elution profile on a calibrated column of S100 Sepharose corresponding to a molecular mass of 64 kDa, even at concentration as low as 10 μ M protein. Separation of the globin chains of each haemoglobin from its accompanying haem group, by h.p.l.c. (Figure 1), followed by quantification of the elution peaks and comparison with an

Figure 1 Globin separation by h.p.l.c.

Samples of adult haemoglobin and the three human embryonic haemoglobins were separated by h.p.l.c. Peak H represents haem. ---, Acetonitrile gradient. The Figure shows an overlay of the chromatographs obtained for separate samples of haemoglobins $\alpha_2\beta_2$, $\alpha_2\epsilon_2$, $\zeta_2\epsilon_2$ and $\zeta_2\gamma_2$.

Figure 2 Oxygen-binding curves of human embryonic haemoglobins

Oxygen-binding curves were measured at a protein concentration of 60 μ M in 50 mM Bistris buffer containing 100 mM NaCl at pH 7.4 and 37 $^{\circ}$ C. The binding curves are shown for $\alpha_2\beta_2$ ($\bullet$), $\zeta_2\epsilon_2$ ($\blacktriangle$), $\alpha_2\epsilon_2$ ($\circ$) and $\zeta_2\gamma_2$ ($\triangle$).

Table 1 Oxygen-binding characteristics of human embryonic and adult haemoglobins

Protein	h^*	$p50$ (mmHg)*	Bohr effect†	2,3-BPG binding‡
$\alpha_2\beta_2$	2.9	9.8	-0.41	0.45
$\alpha_2\epsilon_2$	2.4	5.5	-0.41	0.5
$\zeta_2\epsilon_2$	2.3	3.5	-0.30	0.5
$\zeta_2\gamma_2$	2.3	5.9	-0.30	6.0

* Measurements made at pH 7.4 and 37 $^{\circ}$ C in 50 mM Bistris buffer containing 100 mM NaCl.

† Determined from $\Delta \log p50 / \Delta pH$ in the alkaline region.

‡ Apparent binding constant (mM) obtained from half-saturation point. Abbreviation: 2,3-BPG, 2,3-bisphosphoglycerate.

Figure 3 Effect of 2,3-bisphosphoglycerate concentration on the binding of oxygen to human embryonic haemoglobins

Oxygen-binding curves were determined as described in Figure 2, except that 2,3-bisphosphoglycerate was added to the solutions at the concentrations indicated in the graph. Symbols as in Figure 2.

internal adult haemoglobin standard, showed that each of the globin chains was associated with 1.0 (± 0.1) haem prosthetic groups. N-terminal amino acid sequencing of the ten terminal residues of the α , ϵ and γ chains produced sequences identical with those previously published for these proteins [8–10]. Gas-phase amino acid sequencing of the ζ chain failed to produce any sequence data. However, fast-atom-bombardment m.s. of the tryptic digest of the ζ chain yielded a fragmentation pattern consistent with the presence of an N-terminally acetylated tryptic peptide, as previously reported in the native protein [11,12]. Absorption maxima in the visible absorption spectrum of the carbonmonoxy derivatives of all three embryonic haemoglobins occurred at 419 nm, 540 nm and 564 nm, as reported for the adult protein [13].

Oxygen-binding properties

When oxygen-binding curves were determined under what are normally considered physiological conditions (37 $^{\circ}$ C, pH 7.4, 100 mM NaCl), it was found that each of the human embryonic haemoglobins exhibited a significantly higher affinity for oxygen than did the adult protein (Figure 2). Furthermore, each of the embryonic haemoglobins showed a reasonably high degree of cooperativity, as expressed by their Hill coefficients (all > 2.3 ; Table 1).

Figure 4 The Bohr effect of human embryonic haemoglobins

Oxygen-binding curves were measured in 50 mM Bistris buffers at pH values below 7.0 and in 50 mM Tris buffers at pH values above 7.0. All experiments were performed in the presence of 100 mM NaCl at 37 °C. Symbols as in Figure 2.

When oxygen-binding studies were performed at pH 7.4 in the presence of different concentrations of 2,3-bisphosphoglycerate, the responses of the embryonic haemoglobins were quite different, although under all conditions investigated the embryonic haemoglobins maintained a significantly higher oxygen affinity than did the adult protein under similar conditions (Figure 3).

In the absence of 2,3-bisphosphoglycerate, the three human embryonic haemoglobins show distinct responses in their oxygen affinities to changes in pH, although their degree of co-operativity is essentially unaltered (Figure 4). Furthermore, it should be noted that under neutral pH conditions the $p50$ and Hill coefficient for each of the embryonic haemoglobins were unaltered for measurement made with different protein concentrations in the range 5–150 μM .

DISCUSSION

As outlined above, we have prepared plasmid-based yeast expression systems for the production of human embryonic haemoglobins. Purified samples of each of the three human embryonic haemoglobins Gower I ($\zeta_2\epsilon_2$), Gower II ($\alpha_2\epsilon_2$) and Portland ($\zeta_2\gamma_2$) have been characterized in terms of their general molecular structure. Each protein is a tetramer of the general form $\alpha_2\beta_2$. The tetramers contain four haem groups which, as judged by their spectral characteristics, are bound to the protein in the same way as in the native protein. The N-terminal sequence of the initial ten amino acids has been shown to be as predicted from the gene sequence for the α , γ and ϵ chains. The N-terminal amino acid of the recombinant ζ chain is blocked by an acetyl group, as has been reported to be the case in the naturally occurring molecule. Each embryonic haemoglobin appears to be tetrameric at 60 μM (the protein concentration employed in oxygen-binding studies) and does not appear to be significantly dimerized at concentrations above 10 μM . On the basis of the above information, we can have confidence that the three human embryonic haemoglobins produced in this recombinant process are essentially identical with the naturally occurring proteins, even to the level of post-translational modification.

When considering the functional aspects of these important proteins, it is useful to take into account the studies on naturally occurring mutants and modified adult haemoglobins, from which Perutz and his colleagues have identified four amino acids in the β chain which have a primary role in the binding of 2,3-

bisphosphoglycerate [14–17], namely Val-1, His-2, Lys-82 and His-143. The ϵ chain contains all four of these important amino acids, at the equivalent positions in its sequence, and hence it is not surprising that the embryonic haemoglobins $\zeta_2\epsilon_2$ and $\alpha_2\epsilon_2$ show very similar sensitivity to 2,3-bisphosphoglycerate concentration as does the $\alpha_2\beta_2$ protein, although they possess a higher intrinsic oxygen affinity. The very slightly lower response to 2,3-bisphosphoglycerate seen in the two embryonic proteins containing ϵ chains may well arise from the Leu- β 3–Phe- ϵ 3 substitution, which, Perutz has shown, leads to a slight disturbance in the stereochemistry of the 2,3-bisphosphoglycerate binding provided by the N-terminal amine group of Val-1 [18]. In $\zeta_2\gamma_2$, the γ chain has a His- β 143–Ser- γ 143 substitution, which leads to the loss of two full formal positive charges from the 2,3-bisphosphoglycerate binding site together with the Leu- β 3–Phe- γ 3 substitution outlined above. Thus $\zeta_2\gamma_2$ shows a drastically decreased response to 2,3-bisphosphoglycerate similar to that observed in the fetal haemoglobin, $\alpha_2\gamma_2$ [19].

In the absence of 2,3-bisphosphoglycerate the three human embryonic haemoglobins show distinct responses in their oxygen affinities to changes in pH, although their degree of co-operativity is essentially unaltered. The origins of these differences in response can also be rationalized in terms of the globin amino acid sequences. Although perhaps not universally accepted [see, e.g., [20]] there is wide support for the propositions made by Perutz, Kilmartin and their colleagues [14,21–23] that the Bohr effect arises mainly from the participation of four or five amino acids, with Val- α 1 (NH_2) (25%), His- α 122 (10%), His- β 146 (40%) and Lys- β 82 (20%) contributing to the alkaline Bohr effect and His- β 143 (50%) contributing to the acid Bohr effect. The human embryonic haemoglobin Gower II ($\alpha_2\epsilon_2$) contains all the amino acids detailed above in equivalent positions in its sequence. Thus, apart from its higher intrinsic oxygen affinity, haemoglobin Gower II exhibits a pH response identical with that seen for $\alpha_2\beta_2$. In haemoglobin Portland ($\zeta_2\gamma_2$) there is a Val- α 1 (NH_2)–Ser- ζ 1 (NAC) substitution, which would be predicted to lead to a 25% decrease in the alkaline Bohr effect, and indeed the $\Delta\log p50/\Delta\text{pH}$ value is decreased from -0.41 to -0.30 . This interpretation contradicts the previously presented view, based on limited experimental data and a mis-assignment of the amino acid sequence of the ζ chain, that the decreased alkaline Bohr effect of haemoglobin Portland arises from a substitution at His-122 [24,25]. The γ chain also contains a His- β 143–Ser- γ 143 substitution, and this may well be responsible for the significantly altered acid Bohr effect observed in this protein [25]. The $\zeta_2\epsilon_2$ protein exhibits very high oxygen affinity at all pH values investigated, and thus the estimated Bohr effect is less precise than for $\zeta_2\gamma_2$ or $\alpha_2\epsilon_2$. Nevertheless the human embryonic haemoglobin Gower I ($\zeta_2\epsilon_2$) does show a decreased alkaline Bohr effect consistent with that expected for the Val- α 1 (NH_2)–Ser- ζ 1 (NAC) substitution present in this system. The maintenance of His-143 in the ϵ chain, at least in part, probably leads to the retention of the acid Bohr effect.

We can thus rationalize the experimentally observed allosteric modulation of oxygen binding to the three human embryonic haemoglobins in terms of the actions of specific amino acids, as suggested by Perutz and co-workers. However, the physiological relevance of this potential modulation is difficult to ascertain in the absence of information on the intracellular status of human embryonic pre-erythrocytes. Moreover, the origin of the intrinsically high oxygen affinity of all the embryonic haemoglobins remains to be identified. To this end, we have begun X-ray-diffraction studies on these systems. The $\alpha_2\epsilon_2$ protein has been crystallized in the carbonmonoxy form (in collaboration with Professor E. Baker, Massey University). The tetragonal crystals

diffract to better than 2.5 Å (0.25 nm). A native data set has been collected and analysis has begun.

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