Circe's haemoglobins, pig–human hybrids: functional characterization and structural considerations

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We report the isolation and the functional characterization of α and β chains from pig (*Sus scropha domestica*) haemoglobin, as well as of the pig–human hybrid haemoglobins, $\alpha_2^h\beta_2^p$ and $\alpha_2^p\beta_2^h$ (i.e. Circe's haemoglobins), obtained by mixing the purified α and β pig chains respectively with the corresponding partner human chains. Their functional properties have been compared with those of both parental haemoglobins in order to obtain information on the role of the different subunits and of their inter-relationships, both at the structural and functional levels.

The results indicate that the functional properties of both hybrids are closer to those of the parental haemoglobin that provides the β chains, confirming the major role of the β chains in determining the oxygen affinity and the modulation mechanisms of the tetrameric molecule. This is supported by the thermodynamic properties, since the very low ΔH of oxygen binding that characterizes pig haemoglobin and the $\alpha_2^h\beta_2^p$ hybrid haemoglobin may be taken as the reflection of specific structural properties of pig β chain.

INTRODUCTION

Oxygen transport to tissues is such a vital process that the haemoglobin molecule has evolved to adapt its functional role to extremely variable physiological demands. Thus, haemoglobins are characterized by a number of complex regulatory mechanisms operative at the molecular level and designed to fulfil the physiological requirements of a given species. These mechanisms are based on the thermodynamic connection between the binding of heterotropic effectors (protons, carbon dioxide, chloride ions and organic phosphates) and the reaction with molecular oxygen. Moreover, as outlined in a number of studies [1,2], another feature of the reaction of haemoglobin with oxygen that plays an important role at the physiological level is its temperature-dependence, which is governed by the associated overall enthalpy change (ΔH).

In the modulation of the functional properties of haemoglobin, which is strongly linked to the architecture and the assembly of the subunits, a critical role is played by the inter-subunit α/β contacts, as outlined by the stereochemical model proposed by Perutz [3] on the basis of crystallographic studies [4]. Perturbation of these inter-subunit contacts may be artificially produced either by specific point mutations at the level of one or both subunits or by assembling purified α and β chains from different haemoglobins into hybrid tetramers; in the latter case, the functional characterization of the hybrid haemoglobins may give additional information on the role played by each subunit in determining the functional behaviour of both native and hybrid haemoglobins, as shown for the first time by Antonini et al. [5], employing dog–human haemoglobin hybrids.

Furthermore, much of the experimental work on the comparative aspects of oxygen transport carried out by us in the past few years has been directed toward the understanding of the

structural basis of the low temperature sensitivity (very low exothermic ΔH of oxygen binding) displayed by Arctic, sub-Arctic and other animal haemoglobins. This low temperature sensitivity has great physiological significance, especially with respect to oxygen delivery at the level of peripheral tissues whose temperature may well be lower, under some extreme environmental conditions, than that of the core of the specific organism. In fact, it has been shown that at the molecular level, heat absorption and release can be considered a physiologically relevant modulating factor, similar to heterotropic and homotropic ligands [1,2]. Along this line, an interesting case is represented by pig haemoglobin whose overall ΔH of oxygen binding, measured in the presence of physiological concentrations of 2,3-diphosphoglycerate (2,3-DPG), is markedly lower than that of human haemoglobin under the same experimental conditions, and is comparable with that previously measured for haemoglobin from Arctic species [6]. In order to shed some light on the molecular basis of this phenomenon, we set up a procedure for preparing α and β chains from pig haemoglobin, as well as their respective symmetric hybrids with the corresponding human mate chains ($\alpha_2^h\beta_2^p$ and $\alpha_2^p\beta_2^h$, i.e. Circe's haemoglobins). These particular hybrid haemoglobins have acquired further interest because of recent work on transgenic swine which has been devoted to obtaining an abundant production of human haemoglobin to be used as a potential blood substitute. In fact, the haemolysate from transgenic pigs shows the presence of pig, human and a hybrid haemoglobin formed between human α and pig β globin chains [7]. The other possible symmetric hybrid ($\alpha_2^p\beta_2^h$) is undetectable in all the transgenic pig haemolysates analysed so far [7–9]; in order to understand the reasons for its absence, this hybrid haemoglobin has been assembled *in vitro* via the alloplex intermediate pathway [10].

Here we report the isolation and characterization of α and β

Abbreviations used: 2,3-DPG, 2,3-diphosphoglycerate; P_{50} , oxygen pressure at 50% saturation; HbA, adult human haemoglobin; ΔH , overall heat of oxygenation; PMB, *p*-hydroxymercuribenzoic acid; AUT/PAGE, Acid urea Triton/PAGE.

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chains from pig (*Sus scropha domestica*), as well as of the pig-human hybrid haemoglobins obtained by mixing each of the purified pig globin chains with the corresponding human partner chains. The structural interpretation has been greatly helped by the structure of aquomet porcine haemoglobin obtained at 2.8 Å resolution [11].

MATERIALS AND METHODS

Sample collection and preparation

Fresh blood samples from pig (*Sus scropha domestica*), collected in heparin tubes, were obtained from a slaughterhouse in Cagliari; human blood was provided by informed donors. Cells were washed three times with an iso-osmotic NaCl solution by centrifugation at 1000 *g* and the packed cells were lysed by adding distilled water in a 1:1 ratio. After incubation for 20 min at 4 °C, 1 vol. of CCl₄ was added and the solution was then centrifuged for 10 min at 2500 *g* to remove the ghosts.

Human haemoglobin was dissociated into its chains by the method of Bucci and Fronticelli [12]. The α and β chains were purified according to the method of Geraci et al. [13] and regeneration of the sulphhydryl groups by the procedure of Parkhurst and Parkhurst [14].

Pig haemoglobin was partially dissociated into its chains by incubating at 4 °C for 40 h a 2.5% carbomonoxy pig haemoglobin solution in 0.02 M sodium dihydrogen phosphate/0.1 M NaCl, with a *p*-hydroxymercuribenzoic acid (PMB) solution (8:1 molar ratio to the haemoglobin), previously titrated as described by Bucci and Fronticelli [12], and bringing the pH of the resulting mixture to 4.7 with a few drops of 1 M acetic acid. β chains were purified using a carboxymethylcellulose column (CM-52; Whatman). A preparative column (10 × 20 cm) was used in order to process large volumes of the haemoglobin solution. After dialysis against 10 mM sodium phosphate buffer, pH 6.3 (equilibrated under CO), the haemoglobin-PMB sample was loaded on the CM-52 column, previously equilibrated with the same buffer, and the PMB- β chains were eluted by increasing the pH to 6.7. The SH group (Cys-93) was regenerated according to the procedure of Parkhurst and Parkhurst [14].

To purify the α chains, the pig haemoglobin sample, after incubation with PMB, was diluted 1:1 with 50 mM Tris/HCl buffer, pH 8.9. Under these conditions the solution became very cloudy, and the brownish pellet obtained by centrifugation was solubilized in a small volume of 10 mM HCl (final pH ~ 4.7). The solution, equilibrated under CO, was incubated with sodium dithionite (final concentration 10 mg/ml) for 30 min, and the SH group (Cys-104) was regenerated using dithiothreitol [14]. The solution was then filtered through a Sephadex G25 column equilibrated with 0.1 M sodium phosphate buffer, pH 6.0, and loaded on a DEAE-cellulose column equilibrated with the 10 mM sodium phosphate buffer at pH 8.0, to purify the α chains from the contaminant haemoglobin.

The hybrid haemoglobins were obtained by mixing equimolar amounts of human α with pig β chains ($\alpha_2^h\beta_2^p$) and of pig α with human β chains ($\alpha_2^p\beta_2^h$), assuming equal molar absorptivities at 540 nm. The uncombined chains were always less than 5% of the total tetramer concentration, as determined by densitometric scanning of the gels.

Gel electrophoresis of dissociated globin chains

The purity of the pig chains and hybrid haemoglobins was checked by alkaline PAGE, isoelectric focusing on thin-layer 5% polyacrylamide gel (Ampholine pH range 6–8; Pharmacia LKB

Biotechnology) [15,16] and acid PAGE in 8 M urea and Triton X-100 under denaturing conditions (AUT/PAGE) [17].

Oxygen binding

Oxygen-binding curves were determined by the tonometric method [18] in 0.1 M Mes/Hepes/NaOH buffer + 0.1 M NaCl, both in stripped condition and in the presence of 5 mM 2,3-DPG, at a protein concentration of 1 mg/ml at 20, 25 and 30 °C. Spectrophotometric measurements were carried out with a Cary 2300 spectrophotometer. Concentrated stock solutions of 2,3-DPG were prepared by dissolving the sodium salt of the 2,3-diphosphoglyceric acid (Sigma) in Hepes buffer.

The overall oxygenation enthalpy (J/mol) was calculated from the integrated van't Hoff equation:

$$\Delta H = -2.303R\Delta \log P_{50}[(T_1T_2)/(T_1 - T_2)]$$

where $R = 8.31 \text{ J} \cdot \text{K}^{-1} \cdot \text{mol}^{-1}$ and P_{50} is the oxygen pressure at 50% saturation.

Over the temperature range examined, van't Hoff plots were linear within the experimental error. Confidence limits of the data are $\pm 8\%$ for the P_{50} and $\pm 15\%$ for the ΔH values.

RESULTS

Isolation of α and β pig chains

Figure 1 shows the alkaline PAGE of the purified α and β chains from pig haemoglobin, as obtained by the procedure reported in the Materials and methods section.

Lane 5 in Figure 1 refers to PMB-reacted pig haemoglobin; apart from the undissociated haemoglobin, a new faster-moving (toward the anode) band is clearly discernible. By comparison

Figure 1 Alkaline PAGE

Discontinuous PAGE (pH 8.1–8.9) in Tris/HCl/Tris/glycine of: lane 1, isolated α chains (SH forms); lane 2, mixture of pig haemoglobin and α chains (SH forms); lane 3, isolated pig β chains regenerated in their SH form; lane 4, undissociated pig haemoglobin; lane 5, PMB-reacted pig haemoglobin.

Figure 2 Bohr effect and Hill coefficients

Effect of pH on the oxygen affinity (a) and cooperativity (b) of α (●) and β (○) pig chains. Conditions: 0.1 M Hepes/NaOH buffer + 0.1 M NaCl, 20 °C. The range of error in a single determination of P_{50} values is generally less than 0.05 unit. These values are generally determined in triplicate to minimize the errors in the determinations.

with a sample of PMB-modified adult human haemoglobin (HbA), this band can be taken to represent the PMB-modified pig β chains. The α chains appear to be almost lacking, due to their instability under these conditions (see Materials and methods section).

In the case of α chains, the absorption spectrum (results not shown), between 600 and 500 nm, shows that the haem protein obtained at the end of the purification procedure is in the ferric form. By incubation with carbon monoxide in the presence of sodium dithionite, the α chains are readily converted back into the ferrous derivative.

Properties of isolated globin pig chains

The oxygen-binding properties of the isolated α and β pig chains were determined at 20 °C within the pH range 7.0–8.0, in 0.1 M Hepes/NaOH buffer/0.1 M NaCl (Figure 2). Both pig chains show a very high oxygen affinity and absence of haem–haem interactions, as indicated by the value of the Hill coefficient ($h_{50} \cong 1.0$, Figure 2b). The two pig chains display different oxygen affinities: at pH 7.4, $\log P_{50}$ is -0.60 and -0.15 for α and β chains respectively (Figure 2a). Each of them differs from the corresponding human chain ($\log P_{50}$ of α and β human chains are -0.34 and -0.55 respectively).

Within the pH range examined, the oxygen affinity of both α and β pig chains is almost insensitive to proton concentration (Figure 2a).

The functional properties of the α and β pig chains were studied at 20, 25 and 30 °C. The calculated ΔH is -56.0 kJ/mol of oxygen, after subtraction of the heat of oxygen solubilization for both α and β chains (see Table 1), and does not differ substantially from that reported for the α and β chains of human haemoglobin [19].

Table 1 Oxygen affinity and overall ΔH of oxygen binding (kJ/mol)

Experimental conditions: 0.1 M Hepes buffer plus 0.1 M NaCl at pH 7.4, in the absence (stripped) and in the presence of 5 mM 2,3-DPG; the P_{50} values of musk ox haemoglobin (Hb) (*) are obtained in 0.1 M Tris/HCl buffer + 0.1 M NaCl at pH 7.4, with or without 2,3-DPG.

	$\log P_{50}$		ΔH	
	Stripped	+ 2,3-DPG	Stripped	+ 2,3-DPG
Human HbA	0.43	0.90	–41.0	–36.0
Pig Hb	0.69	1.11	–34.0	–17.0
$\alpha^h\beta^p$ Hb	0.70	1.13	–23.8	–13.4
$\alpha^p\beta^h$ Hb	0.28	0.75	–36.0	–32.2
Pig β chain	–0.13	–0.35	–56.0	–
Pig α chain	–0.57	–0.59	–56.0	–
Reindeer Hb ¹	0.90	1.07	–14.0	–14.0
Musk ox Hb ²	1.27*	1.27*	–15.0	–15.0

¹ Values from [23,24].

² Values from [24,25].

Figure 3 AUT/PAGE

AUT/PAGE of dissociated globin chains of: lane 1, $\alpha_2\beta_2^p$; lane 2, pig; lane 3, $\alpha_2\beta_2^h$; lane 4, human haemoglobins. Conditions: 5% acetic acid/8 M urea in the presence of Triton X-100.

Functional properties of the $\alpha_2^h\beta_2^p$ and $\alpha_2^p\beta_2^h$ hybrids (Circe's haemoglobins)

The homogeneity of hybrid haemoglobins, as well as their subunit composition, is clearly demonstrated by the AUT/PAGE (Figure 3).

The oxygen-binding properties of hybrid haemoglobins were determined at 20 °C as a function of pH (6.0–8.0) in 0.1 M Mes/Hepes/0.1 M NaCl buffer, both in the absence (Figure 4a) and in the presence (Figure 4b) of 5 mM 2,3-DPG. The hybrid molecules bind oxygen cooperatively at all pH values, the Hill coefficient (h_{50}) always being within the range 2.2–2.6.

The oxygen-affinity of the hybrid molecule $\alpha_2^h\beta_2^p$ is similar to that of native pig haemoglobin within the pH range examined, and lower than that of human haemoglobin. The Bohr effect is identical with that of pig haemoglobin and slightly lower than that of human haemoglobin ($\Delta \log P_{50}/\Delta \text{pH} = -0.60 \pm 0.02$ and

Figure 4 Bohr effect
Effect of pH on the oxygen affinity of $\alpha_2^h\beta_2^p$ (■), $\alpha_2^p\beta_2^h$ (●), pig (□) and human (○) haemoglobins in the absence (a) and in the presence (b) of 5 mM 2,3-DPG. Conditions: 0.1 M Mes/Hepes/NaOH buffer plus 0.1 M NaCl at 20 °C. The range of error in a single determination of P_{50} values is generally less than 0.05 unit. These values are generally determined in triplicate to minimize the errors in the determinations.

-0.70 ± 0.03 for pig and human haemoglobin respectively in the pH range 6.5–8.0).
The decrease in oxygen affinity observed upon addition of 5 mM 2,3-DPG is similar to, if not identical with, that measured under the same conditions for native pig haemoglobin (at pH 7.5 $\Delta\log P_{50} = -0.43$, Figure 4b). The homotropic and heterotropic modulations of this hybrid haemoglobin molecule are clearly similar to those of native pig haemoglobin.
The oxygen affinity of the $\alpha_2^p\beta_2^h$ hybrid haemoglobin is much higher than that of pig and the $\alpha_2^h\beta_2^p$ hybrid haemoglobin under all the experimental conditions used. At 20 °C and pH 7.4, in the absence of 2,3-DPG, $\log P_{50} = 0.30$ and 0.43 for the $\alpha_2^p\beta_2^h$ hybrid and HbA respectively and 0.70 for pig and $\alpha_2^h\beta_2^p$ haemoglobin (Figure 4a). The alkaline Bohr effect of the $\alpha_2^p\beta_2^h$ hybrid is slightly lower than that of pig and the $\alpha_2^h\beta_2^p$ haemoglobin ($\Delta\log P_{50}/\Delta\text{pH} = -0.52 \pm 0.02$ calculated in the pH range 6.5–8.0). The organic phosphate effect is similar to that for pig and the $\alpha_2^h\beta_2^p$ hybrid, the $\Delta\log P_{50}$ between stripped conditions and in the presence of 5 mM 2,3-DPG, measured at pH 7.4, being -0.43 (Figure 4b).
An interesting finding emerges from the comparison of the ΔH values of the hybrid molecules with those of both parental haemoglobins and Arctic and sub-Arctic mammals (Table 1).

DISCUSSION
Adult pig haemolysate contains one haemoglobin component whose primary structure has been determined [20]. Comparison of the amino acid sequence of α and β pig globins with the corresponding human globins shows 22 substitutions for each globin. None of these substitutions involves the $\alpha_1\beta_2$ interface, which is, therefore, entirely conserved between pig and human haemoglobins. On the contrary, there are 13 amino acid substitutions at $\alpha_1\beta_1$ intersubunits contacts (Table 2), seven of which are on the β and six on the α subunits. In particular, the $\beta 112$ cysteine of HbA is substituted in pig haemoglobin by valine. The absence of this cysteine probably explains why the conditions used to dissociate the human haemoglobin tetramer into α and β chains [12] are not applicable to pig haemoglobin.
The stability of isolated α and β chains from pig haemoglobin allowed a functional characterization as a function of pH and temperature. They show a very high oxygen affinity and absence of cooperativity (see Figure 2). It should be noted, however, that the P_{50} of pig α chain is 1.8 times lower than that of human α chains and shows no significant pH dependence, whereas that of pig β chains is 2.5 times higher than that of human β chains

Table 2 Amino acid differences between pig and human α and β chains in the positions involved in $\alpha_1\beta_1$ contacts in deoxyHbA

β chain			$\alpha_1\beta_1$ contacts deoxy	α chain	
pig	human	human			
51	A	P		H	122
				A	120
112	V	C		P	119
				T	118
				F	117
116	R	H		E	116
				A	115
117	R	H		P	114
				L	113
120	H	L		A	111
				A	110
123	N	T		V	107
				L	106
125	D	P		H	103
				L	34
				R	31
				E	30

α chain			$\alpha_1\beta_1$ contacts deoxy	β chain	
pig	human	human			
35	G	S		Q	131
				A	129
113	H	L		A	128
115	D	A		Q	127
116	D	E		P	124
118	N	T		H	116
				P	51
120	S	A		V	33
				R	30

under the same experimental conditions. Moreover, the overall ΔH of oxygen binding to α and β pig chains, measured within the temperature range 20–30 °C, is strongly exothermic (Table 1), comparable with that observed in human α and β chains [19] and much higher (in absolute terms) than that of the whole pig haemoglobin.

Preparation of the hybrid tetramers made it possible to test the role played by their subunit interfaces in the allosteric and thermodynamic properties. The overall functional properties of the two hybrids are very different: those of the $\alpha_2^h\beta_2^p$ hybrid haemoglobin are almost identical with those of native pig haemoglobin, whereas those of the hybrid $\alpha_2^p\beta_2^h$ are similar to HbA, suggesting a major role of the β chains in determining the oxygen affinity and the modulation mechanisms that operates in the hetero-tetrameric molecule. This also applies to the thermodynamic properties of both hybrid molecules: in fact, under physiological conditions (pH 7.4 in the presence of 5 mM 2,3-DPG) the $\alpha_2^h\beta_2^p$ hybrid haemoglobin shows a low ΔH of oxygenation, similar to that of pig haemoglobin, whereas the $\alpha_2^p\beta_2^h$ hybrid haemoglobin shows a much higher ΔH , similar to that of HbA. Hence, the very low ΔH of oxygen binding that characterizes the $\alpha_2^h\beta_2^p$ hybrid haemoglobin could be taken as the reflection of some peculiar structural properties of the β pig chains. In this respect, recent papers on the three-dimensional structure of aquomet porcine [11] and bovine haemoglobins [21], together with previous work on the functional characterization of pig haemoglobin [6], allow some stimulating considerations. Perutz et al. [21] explain the lower intrinsic oxygen affinity of bovine haemoglobin (compared with human haemoglobin) on the basis of structural differences at the level of the N-terminal residues and the A helix, which in bovine β chains are shifted closer to the main body of the subunit and the dyad axis of symmetry. Interestingly, a comparable contraction of the N-terminus and helix A toward the molecular dyad axis has been observed as the main structural aspect that differentiates pig β chains from the corresponding human subunits [11]. The results obtained on Circe's haemoglobins seem to give further support to the hypothesis advanced by Perutz et al. [21]: in fact the $\alpha_2^h\beta_2^p$ hybrid haemoglobin has a lower oxygen affinity compared with HbA, whereas the $\alpha_2^p\beta_2^h$ hybrid haemoglobin, in which the contraction of the N-terminus is not present, has a higher oxygen affinity.

Another interesting functional difference between pig and human haemoglobin concerns the markedly reduced oxygenation enthalpy value displayed by pig haemoglobin in the presence of 2,3-DPG compared with that of human HbA under the same conditions (see Table 1). Under stripped conditions, i.e. in the absence of organic phosphates, native pig haemoglobin displays an overall ΔH of oxygenation very similar to that of human haemoglobin. Upon addition of physiological concentrations of 2,3-DPG, the thermodynamic behaviour of the two proteins diverges greatly, and the overall oxygenation enthalpy of pig haemoglobin decreases dramatically, becoming comparable with that of arctic ruminants [22]. The results obtained on Circe's haemoglobins allow some additional conclusions which are reported below.

Under stripped conditions, the ΔH of $\alpha_2^h\beta_2^p$ haemoglobin is about 30 % lower than that of native pig haemoglobin; upon addition of 2,3-DPG in saturating amounts, a further decrease of about 45 % is observed and the ΔH reaches a value that is even lower, by about 20 %, than that of pig haemoglobin in the presence of 2,3-DPG. On the whole, the $\alpha_2^h\beta_2^p$ haemoglobin, in the presence of organic phosphate, shows a 60 % decrease in the exothermic enthalpy of oxygen binding in comparison with the native pig haemoglobin in the absence of 2,3-DPG (Table 1). On

Table 3 Amino acid substitutions present at the $\alpha_1\beta_1$ interface of β chains of human, cow, reindeer and pig haemoglobin

Identical substitutions present in the haemoglobins with low enthalpy of oxygenation with respect to HbA are highlighted in bold.

	Position of amino acid substitution						
	51	112	116	117	120	123	125
Human	P	C	H	H	K	T	P
Cow	A	V	R	N	K	T	V
Reindeer	A	V	R	N	K	T	V
Pig	A	V	R	R	H	N	D

the other hand, the $\alpha_2^p\beta_2^h$ shows ΔH values very similar to those of HbA (10 % lower), and no significant reduction in ΔH upon addition of 2,3-DPG (Table 1), apart from the endothermic contribution due to the release of the 2,3-DPG from the oxyhaemoglobin.

These findings indicate significant differences in the thermodynamic properties of the two hybrid haemoglobins and significant similarities between each hybrid and the native parental haemoglobin that provides the β chains. All together the functional data indicate that one or more of the substitutions present in the $\alpha_2^h\beta_2^p$ hybrids at the level of the $\alpha_1\beta_1$ interface play a role in determining the very low ΔH of oxygen binding of this hybrid molecule. In particular, the substitutions observed at the $\alpha_1\beta_1$ interface in the $\alpha_2^h\beta_2^p$ compared with human haemoglobin are: Pro-51 β → Ala, Cys-112 β → Val, His-116 β → Arg, His-117 β → Arg, Lys-120 β → His, Thr-123 β → Asn, Pro-125 β → Asp (see Table 2). By comparison of the amino acid sequences of β chains from pig, cow and reindeer haemoglobins, all of which display a low ΔH of oxygen binding compared with human haemoglobin (Table 3), the most reasonable interpretation is that the substitutions which may play a role in determining the low ΔH of oxygen binding in these haemoglobins are those involving residues 51, 112 and 116 of the β chains.

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