The 'natural' hybrid haemoglobin from mule

Interrelationships with its parent haemoglobins from horse and donkey

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The equilibrium O₂-binding properties of the hybrid haemoglobin (Hb) present *in vivo* in erythrocytes from mule and of its parent Hbs from horse and donkey were compared with special reference to the effect of heterotropic ligands such as Cl⁻, D-glycerate 2,3-bisphosphate (DPG) and inositol hexakisphosphate. All these Hbs display a decreased effect by polyphosphates, confirming that what has been observed for horse Hb [Giardina, Brix, Clementi, Scatena, Nicoletti, Ciccetti, Argentin & Condò (1990) *Biochem. J.* **266**, 897–900] is common to other equine species, at least from a qualitative standpoint. However, different quantitative aspects can be detected, which can be accounted for by a different role for the two types of chain in characterizing the binding free energy for the various heterotropic effectors. In particular, it is shown that the binding mode of DPG and inositol hexakisphosphate displays different features since long-range effects can be observed clearly for inositol hexakisphosphate but not for DPG. In general terms, in spite of a different intrinsic O₂ affinity, the modulation of functional properties by third ligands leads these Hbs to behave, under physiological conditions, similarly to human HbA. It might represent an interesting example of how different species with similar functional needs find different ways to produce a similar functional behaviour.

INTRODUCTION

Mammals display a wide range of whole-blood O₂ affinity, mostly dependent on three factors: the intrinsic O₂ affinity of haemoglobin (Hb), the concentration of intraerythrocytic D-glycerate 2,3-bisphosphate (DPG) and the response of Hb to DPG and Cl⁻.

As far as the DPG effect is concerned, mammalian Hbs have been broadly divided into two groups: those with an intrinsically high O₂ affinity, modulated in the erythrocytes by interaction with DPG, and those that are almost insensitive to the presence of this effector, showing an intrinsically low O₂ affinity.

Hbs from ruminants and felids have been reported as being examples of the second group, and those from man, horse, rabbit and rat are examples of the first (Perutz & Imai, 1980; Perutz, 1983). However, this distinction was mainly based on experiments carried out in the presence of a high concentration of Cl⁻ without considering the interrelationships between this anion and the organic phosphates. In fact, as demonstrated for some ruminants, Cl⁻, at physiological concentration, may completely replace DPG in regulating O₂ affinity of Hb (Fronticelli *et al.*, 1988; Giardina *et al.*, 1990b).

Hence, in order to re-evaluate the above classification, we have recently investigated the functional properties of horse Hb, which had been described as a high-affinity Hb with a normal reactivity towards DPG (Bunn & Kitchen, 1973). The results showed that this Hb is much less sensitive to organic phosphates than previously described, displaying an intrinsic low O₂ affinity and being characterized by a small effect of DPG (Giardina *et al.*, 1990a).

This phenomenon prompted us to carry out a more detailed study on the functional properties of horse Hb with special regard to the effect of Cl⁻ concentration. Furthermore, we have characterized the Hb from donkey and that from mule, the sterile hybrid between horse and donkey, in order to investigate whether the functional behaviour observed in horse Hb may be a general mechanism for modulation of Hb function in equines or if it may be linked to some peculiar physiological needs of the animal.

Moreover, since for mule Hb the α - and β -chains must originate from the parent Hbs, such a system might display some hybrid component, with α -subunit from one parent and β -subunit from the other. Therefore, selecting the appropriate component of mule Hb, one can compare its functional properties with those of either one of the parent Hbs, in order to obtain information on the role of the different subunits in the ligand-linked heterotropic

Table 1. Relative proportions and isoelectric points of Hb components of horse, donkey and mule

Species	Component	Composition (%)	pI
Horse	Hb1	80	6.6
	Hb2	20	6.8
Donkey	Hb	100	6.7
Mule	Hb1	20	6.6
	Hb2	80	6.8

Abbreviations used: DPG, D-glycerate 2,3-bisphosphate; P_{50} , pressure required to give 50% saturation; Hb, haemoglobin; HbA, normal human adult haemoglobin.

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effects and in the influence on the intersubunit functional interrelationships.

This possibility is of great importance since it is well known that the intersubunit contacts play a key role in regulating several functional properties of Hb, as clearly outlined by the stereochemical model proposed by Perutz on the basis of crystallographic studies (Perutz, 1970; Baldwin, 1980) and by the data reported on the functional properties of hybrid Hbs obtained from human HbA and *Xenopus laevis* Hb (Condò *et al.*, 1987). Therefore mule may represent an elegant way of obtaining *in vivo* a hybrid Hb that can give additional information on the role played by each subunit in determining the functional behaviour of both native and hybrid (or hybrids) Hbs.

MATERIALS AND METHODS

Blood samples from horse, donkey and mule were collected from the jugular vein without anaesthesia into an iso-osmotic NaCl solution containing 2 mM-EDTA. The cells were washed three times with iso-osmotic NaCl solution by centrifugation at 1000 *g*, and the packed cells lysed by adding 2 vol. of cold distilled water. The stroma were removed by centrifugation at 12000 *g* for 30 min.

Analytical isoelectric focusing on polyacrylamide gels and electrophoresis on cellulose acetate films in 6 M-urea were performed as previously described (Condò *et al.*, 1987); in the latter case, samples were composed of globin obtained by the HCl/acetone method (Rossi-Fanelli *et al.*, 1958) and dissolved in the gel buffer.

Stripped Hb was obtained by passing the haemolysate first through a Sephadex G-25 column, equilibrated with 0.01 M-Tris/HCl buffer, pH 8.0, containing 0.1 M-NaCl, and afterwards through a column of mixed-bed ion-exchange resin (Bio-Rad AG 501 X8) equilibrated with Cl⁻-free 0.01 M-Hepes/NaOH buffer, pH 7.4. Inositol hexakisphosphate and DPG were purchased as the sodium salts from Sigma Chemical Co.

Purification of Hb components from horse and mule haemolysates was carried out as follows. Haemolysate, dialysed against 0.01 M-potassium phosphate buffer, pH 6.5, was applied to a Whatman CM-52 CM-cellulose column (3 cm × 35 cm) equilibrated with the same buffer. Elution was achieved by increasing the ionic strength with NaCl gradients starting from the equilibrating buffer. The temperature was kept at 4 °C and the flow rate at 120 ml/h.

O₂-dissociation curves were determined spectrophotometrically by the tonometric method (Giardina & Amiconi, 1981). Curve-fitting as a function of effector concentration was carried out on a PDP II/23 (Digital Equipment, U.S.A.) by using a non-linear least-squares fitting procedure with a Marquardt algorithm according to the following equation:

$$\log P_{50}^{\text{obs.}} = \log P_{50}^0 + R \cdot \log \{ (1 + K_d \cdot [E]) / (1 + K_0 \cdot [E]) \} \quad (1)$$

where $\log P_{50}^{\text{obs.}}$ refers to the O₂ affinity observed at a given concentration [E] of the effector under investigation, $\log P_{50}^0$ corresponds to the O₂ affinity displayed in the absence of the effector, K_d and K_0 are the association equilibrium constants for the effector to unliganded and liganded Hb respectively and R corresponds to the number of effector binding sites per haem group (i.e. $R = 1$ for Cl⁻ and $R = 0.25$ for DPG and inositol hexakisphosphate).

The use of P_{50} instead of P_m is justified by the fact that, owing to the highly symmetrical shape of the binding curves, $P_m \approx P_{50}$. Furthermore, in view of the low Hb concentration and the relatively low affinity of organic phosphates and Cl⁻ for Hb under the solvent conditions used, free effector concentration

may be approximated to the total effector concentration. Finally, the linkage relations were used considering the activities of all other components of the system to be constant.

RESULTS

As previously reported (Trujillo *et al.*, 1967), the electrophoretic analysis performed on equine blood samples show the presence of only one Hb component in donkey haemolysate, whereas those from horse and mule are both characterized by the presence of two different Hb components. The relative proportions and the isoelectric points of all the components are reported in Table 1.

As far as the subunit composition is concerned, electrophoresis of the globins in 6 M-urea at pH 8.6 shows that donkey Hb is characterized by only one type of α -chain whereas horse and mule Hbs are characterized by two, one displaying the same mobility as the donkey α -chain, the other one being slower. In horse Hb the slower α -subunit corresponds to the prevailing component (approx. 80%), and in mule Hb the relative proportions are reversed (80% for the fast and 20% for the slow α -chain).

These observations suggest that the α -chain of the major component from mule Hb is closely similar to that from donkey Hb, whereas for the smaller component the α -chain is more similar to that from horse.

For all the Hbs β -chains display the same electrophoretic mobility, which is slower than that of human β -chains. However, a fingerprint analysis (results not shown) has produced evidence that β -chains from the major component of mule Hb are virtually identical with those from the major component of horse Hb, whereas significant differences have been observed with respect to donkey β -chains. Therefore it looks as if the major component of mule Hb may have α -chains from donkey Hb and β -chains from horse Hb, whereas the opposite is probably true for the minor component.

Fig. 1. O₂ Bohr effect of horse Hb at 20 °C

○, Stripped Hb; ●, plus 3 mM-DPG; △, plus 3 mM-inositol hexakisphosphate. Broken lines refer to HbA stripped and in the presence of 3 mM-DPG or -inositol hexakisphosphate (going from the bottom to the top). Conditions: 0.1 M-Tris or -Bistris buffer plus 0.1 M-NaCl.

Fig. 2. O₂ Bohr effect of donkey Hb at 20 °C

○, stripped Hb; ●, plus 3 mM-DPG; △, plus 3 mM-inositol hexakisphosphate. Broken lines refer to HbA stripped and in the presence of 3 mM-DPG or -inositol hexakisphosphate (going from the bottom to the top). Conditions: 0.1 M-Tris or -Bistris buffer plus 0.1 M-NaCl.

Fig. 3. O₂ Bohr effect of mule Hb at 20 °C

○, stripped Hb; ●, plus 3 mM-DPG; △, plus 3 mM-inositol hexakisphosphate. Broken lines refer to HbA stripped and in the presence of 3 mM-DPG or -inositol hexakisphosphate (going from the bottom to the top). Conditions: 0.1 M-Tris or -Bistris buffer plus 0.1 M-NaCl.

The Hb components of these haemolysates were separated by ion-exchange chromatography, and the functional characterizations reported below for horse and mule Hbs refer to the major component of both Hb systems. Therefore, throughout this work, mule Hb refers to the component in which α -chains seem to derive from donkey Hb and β -chains from horse Hb.

Figs. 1, 2 and 3 report the pH-dependence of O₂ affinity for horse Hb (Fig. 1), donkey Hb (Fig. 2) and mule Hb (Fig. 3) in

Fig. 4. Effect of Cl⁻ (□), DPG (○ and ●) and inositol hexakisphosphate (△) concentration on the O₂ affinity of horse Hb (a), donkey Hb (b) and mule Hb (c) in 0.1 M-Hepes buffer, pH 7.4-7.5, at 20 °C

The effect of DPG concentration was measured both in the absence (○) and in the presence (●) of 0.1 M-NaCl, and that of inositol hexakisphosphate only in the presence of 0.1 M-NaCl.

the absence (stripped conditions) and in the presence of either 3 mM-DPG or -inositol hexakisphosphate. The first point to make is that at every pH value all equine Hbs display an O₂ affinity (in the presence of 0.1 M-Cl⁻) significantly lower than that of HbA. However, addition of DPG brings about a decrease in O₂ affinity (expressed as an increase in log P₅₀) that is smaller for equine Hbs than for human HbA, this general phenomenon being in line with the behaviour reported previously for horse Hb (Giardina *et al.*, 1990a). Thus the overall effect of Cl⁻ and DPG is such that, under physiological conditions (i.e. in the presence of Cl⁻ and DPG at pH 7.4), the O₂ affinity of equine Hbs approaches that of HbA.

A decreased functional effect is also observed with inositol hexakisphosphate, and it is especially evident for mule Hb (see Fig. 3). In this respect it is important to mention that our experimental data on the interaction of inositol hexakisphosphate with horse Hb may account for the higher O₂ affinity displayed by horse erythrocytes after incorporation of inositol hexakisphosphate as compared with similarly treated human erythrocytes (Bailleul *et al.*, 1989).

In order to have a more quantitative look at the interplay between O₂ affinity and binding of heterotropic effectors, such as Cl⁻, DPG and inositol hexakisphosphate, we carried out, for all

Table 2. Binding parameters, expressed as M^{-1} , for different effectors (indicated in parentheses) to equine haemoglobins, as calculated from data reported in Fig. 4

Conditions: 0.1 M-Hepes buffer, pH 7.5, at 20 °C. The s.d. of all the parameters was approx. $\pm 10\%$.

Parameter	Horse	Donkey	Mule
K_d (Cl^-)	17.5	31.5	28.7
K_0 (Cl^-)	2.3	4.0	2.2
K_d (DPG)	3.8×10^4	2.2×10^5	5.0×10^4
K_0 (DPG)	3.2×10^2	1.8×10^3	5.0×10^2
K_d (DPG + Cl^-)*	3.8×10^4	1.5×10^4	2.3×10^4
K_0 (DPG + Cl^-)*	3.2×10^2	1.0×10^3	1.3×10^3
K_d (inositol hexakisphosphate + Cl^-)*	1.2×10^6	7.2×10^5	6.0×10^4
K_0 (inositol hexakisphosphate + Cl^-)*	5.2×10^2	8.7×10^2	4.2×10^2

* The binding parameters refer to the first effector (in parenthesis) in the presence of a constant concentration (0.1 M) of the second effector.

three Hbs, a detailed investigation on the dependence of O_2 affinity on the concentration of the various effectors. This dependence is presented in Fig. 4 for horse Hb (Fig. 4a), donkey Hb (Fig. 4b) and mule Hb (Fig. 4c), and the parameters obtained from eqn. (1) for each effector, i.e. Cl^- , DPG (in the absence and in the presence of Cl^-) and inositol hexakisphosphate (in the presence of Cl^-), are reported in Table 2.

The parameters in Table 2 allow several functional considerations, namely: (i) the effect of Cl^- is very similar in the three cases; (ii) donkey Hb, which among the three equine Hbs shows the lowest intrinsic O_2 affinity in the absence of effectors, displays the highest affinity for DPG both in the deoxy form and in the oxygenated form; (iii) a direct functional interplay between Cl^- and DPG is observed to a meaningful extent again only in donkey Hb (as from the 10-fold decreased affinity for DPG in the unliganded form in the presence of 0.1 M- Cl^-); (iv) a decreased but peculiar functional interplay between Cl^- and DPG is observed for mule Hb since the presence of 0.1 M- Cl^- slightly decreases the affinity of DPG for the unliganded form but enhances its affinity for the liganded form, thus bringing about a decreased DPG-linked $\Delta \log P_{50}$ (see Fig. 4c); (v) any functional interplay between Cl^- and DPG disappears in horse Hb, which shows the same affinity for DPG in both the absence and presence of Cl^- ; (vi) the affinity of inositol hexakisphosphate (measured in the presence of Cl^-) is essentially the same for the liganded form of all three equine Hbs, whereas large differences can be observed for deoxyHbs, the highest affinity being displayed by horse Hb and a particularly low affinity being shown by mule Hb.

The overall picture emerging from this functional analysis is not simple and indeed reflects the complexity of the various functional interplays between different heterotropic ligands. Moreover, it is crucial to point out that the component of mule Hb under investigation indeed displays functional properties markedly different from those of either one of the parent Hbs. This strongly reinforces the evidence that this component is a hybrid, and independent structural observations suggest that it is composed of α -chains from donkey Hb and β -chains from horse Hb.

DISCUSSION

The co-operative mechanism of ligand binding to Hb has been shown to be mostly related to alterations in the stereochemistry

of subunit interfaces (Perutz, 1970). Therefore special attention has been addressed to functional studies of hybrid Hbs in which the interaction between the two types of subunit were functionally and/or structurally altered with reference to the native molecule (Nagai, 1977; Imai *et al.*, 1980; Condò *et al.*, 1987).

Along these lines, we have carried out an investigation on the Hb component from mule, a 'natural' hybrid molecule, comparing the results with the parent Hbs from horse and donkey. The three equine Hbs indeed display clear differences both in terms of O_2 binding and the interaction with heterotropic effectors, especially at the level of the unliganded form.

In fact, meaningful differences in the interaction with heterotropic effectors can be observed in donkey Hb as compared with other equine Hbs, namely (i) it is characterized by the largest affinity (by about one order of magnitude) for DPG both in the deoxy form and in the oxygenated form, and (ii) only in this Hb do Cl^- ions seem to compete efficiently (at least in the unliganded form) with DPG.

Thus in horse Hb no influence can be detected between the two effectors and in mule Hb this interplay if any is very small. As a whole, these features suggest significant structural differences between donkey Hb on the one hand and horse and mule Hbs on the other with respect to the stereochemistry of the DPG-binding pocket, which may also represent the structural background for the different interplay between DPG and Cl^- . This structural alteration might also be correlated with the lower intrinsic O_2 affinity displayed by donkey Hb (see Fig. 4b), this being indicative of a T quaternary conformation (Monod *et al.*, 1965) that is energetically more stable for donkey Hb than for other equine Hbs. It is worth pointing out that, from the amino acid sequence, only a very limited number of substitutions have been detected between horse and donkey Hbs (Garrick & Garrick, 1983). In particular, the β -chain has been reported to differ at positions 52 and 87 where the donkey has alanine and glutamine respectively and horse glycine and alanine. The α -chain of the donkey has Asn-20, Phe-24, Lys-60 and Thr-131, whereas the horse has His-20, Tyr-24 or Phe-24, Gln-60 or Lys-60 and Ser-131.

Furthermore, an important observation coming from the dependence of O_2 affinity of equine Hbs on effector concentration concerns the different trend observed for DPG and inositol hexakisphosphate. Thus horse and mule Hbs display a similar affinity for DPG in both the deoxy form and the oxygenated form, whereas in the case of inositol hexakisphosphate the unliganded form of horse Hb displays an approx. 20-fold higher affinity than mule Hb. All three equine Hbs, in the liganded form, display similar affinity for inositol hexakisphosphate, indicating significant differences for the binding of the two effectors only in the unliganded form. Therefore in the following discussion we shall focus our attention primarily on the effector-binding parameters to the unliganded forms of the three equine Hbs.

A first important point concerns the close similarity for DPG binding to the unliganded form of horse and mule Hb (Table 2), which is about one order of magnitude smaller than in donkey Hb. This result is perfectly in line with the fact that in the mule Hb component under investigation the β -chains, which are the ones supposed to contribute to the formation of the DPG-binding pocket, are from horse (Fig. 3).

The opposite behaviour is observed when comparing the affinity binding constant of inositol hexakisphosphate to horse and donkey Hbs (Table 2). Such a finding can be accounted for by assuming that, although the same residues are responsible for the interaction with both effectors, the stereochemistry of the binding site is suited to a different extent to the two effectors, underlying a different mode of interaction for the two molecules.

Further elucidation of the structural basis of the functional

difference for interaction of the two effector molecules can be obtained from the striking observation that the affinity of mule Hb for inositol hexakisphosphate is much lower than in horse and donkey Hbs, displaying a similar value for DPG and inositol hexakisphosphate binding (see Table 2). Such unexpected behaviour suggests that this difference between horse and mule Hbs, which share the same β -chains and display similar DPG-binding properties, must originate from the α -chains, which in mule Hb are like those of donkey Hb. In fact, these features indicate that the binding site for inositol hexakisphosphate is significantly different from that for DPG and might extend its functional influence through the $\alpha_1\beta_2$ (or $\alpha_2\beta_1$) subunit interface. Therefore the highest affinity of inositol hexakisphosphate for horse Hb could imply that α -chains from horse Hb somehow provide a free-energy contribution to inositol hexakisphosphate binding that is not undertaken by α -chains from donkey (present in mule Hb). Obviously, the additional free-energy contribution for inositol hexakisphosphate binding to horse Hb can be attributed to either (i) some additional residue present in α -chains from horse Hb and/or (ii) a different stereochemical arrangement at the α/β interface. Whatever is the case, such an occurrence would explain why in both donkey Hb and mule Hb the affinities for DPG and inositol hexakisphosphate are closely similar, the higher affinity of donkey Hb for both effectors being only related to a more favourable interaction with polyphosphate at the level of β -subunits (which are different from those of mule Hb).

It may be important to point out that α -chains from donkey Hb display a substitution with respect to horse Hb at residue H14 α (serine in horse Hb and threonine in donkey Hb). This residue, which is also present in HbA, has been proposed, on the basis of structural and functional considerations (O'Donnell *et al.*, 1979; Nigen *et al.*, 1980), to be important for the formation of an anion-binding site in HbA together with the *N*-terminal residue Val-1 α and the *C*-terminal 141 α . It might also be a potential candidate as an additional binding site for one of the phosphate groups of inositol hexakisphosphate and such a role could also be played in horse Hb (where the same residues are present). On the other hand, its substitution with threonine at position 131 α in donkey Hb might be responsible for a perturbation of the inositol hexakisphosphate interaction in donkey Hb and in the component of mule Hb under investigation where α -chains are derived from donkey.

On the whole and in qualitative terms, the behaviour observed for horse Hb (Giardina *et al.*, 1990a) seems to be fairly general of equine Hbs even though the quantitative aspects may differ

from species to species. In this respect, very important and quite novel evidence emerging from this investigation concerns a clear-cut difference for the interaction of these Hbs with effectors such as Cl⁻, DPG and inositol hexakisphosphate.

In conclusion, a very interesting physiological aspect is presented in that, in spite of intrinsic functional behaviour that is significantly different from HbA, these equine Hbs display various modulation effects, induced by third ligands, such that under physiological conditions their functional properties become much more similar to those shown by HbA. This indeed represents an interesting example of how Hbs from different species but living in closely similar environments find different ways to produce a similar functional behaviour.

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REFERENCES

- Bailloul, C., Borrelly-Villereal, M. C., Chassaigne, M. & Ropars, C. (1989) *Biotechnol. Appl. Biochem.* **11**, 31–40
- Baldwin, J. M. (1980) *J. Mol. Biol.* **136**, 103–128
- Bunn, H. F. & Kitchen, H. (1973) *Blood* **42**, 471–479
- Condò, S. G., Giardina, B., Bellelli, A. & Brunori, M. (1987) *Biochemistry* **26**, 6718–6722
- Fronticelli, C., Bucci, E. & Razynska, A. (1988) *J. Mol. Biol.* **202**, 343–348
- Garrick, M. D. & Garrick, L. M. (1983) in *Red Blood Cells of Domestic Mammals* (Agar, N. S. & Board, P. G., eds.), pp. 165–207, Elsevier Science Publishers, Amsterdam
- Giardina, B. & Amiconi, G. (1981) *Methods Enzymol.* **76**, 417–427
- Giardina, B., Brix, O., Clementi, M. E., Scatena, R., Nicoletti, B., Cicchetti, R., Argentin, G. & Condò, S. G. (1990a) *Biochem. J.* **266**, 897–900
- Giardina, B., Brix, O., Colosimo, A., Petruzzelli, R., Cerroni, L. & Condò, S. G. (1990b) *Eur. J. Biochem.* **194**, 61–65
- Imai, K., Ikeda-Saito, M., Yamamoto, H. & Yonetani, T. (1980) *J. Mol. Biol.* **138**, 635–648
- Monod, J., Wyman, J. & Changeux, J.-P. (1965) *J. Mol. Biol.* **12**, 88–118
- Nagai, K. (1977) *J. Mol. Biol.* **111**, 41–53
- Nigen, A. M., Manning, J. M. & Alben, J. O. (1980) *J. Biol. Chem.* **255**, 5525–5532
- O'Donnell, S., Mandaro, R., Schuster, T. M. & Arnone, A. (1979) *J. Biol. Chem.* **254**, 12204–12210
- Perutz, M. F. (1970) *Nature (London)* **229**, 421–426
- Perutz, M. F. (1983) *Mol. Biol. Evol.* **1**, 1–28
- Perutz, M. F. & Imai, K. (1980) *J. Mol. Biol.* **136**, 183–191
- Rossi-Fanelli, A., Antonini, E. & Caputo, A. (1958) *Arch. Biochem. Biophys.* **30**, 608–615
- Trujillo, J. M., Walden, B., O'Neil, P. & Anstall, H. B. (1967) *Nature (London)* **270**, 88–90

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