Adult and fetal haemoglobin J-Sardegna [$\alpha 50(\text{CE}8)\text{His} \rightarrow \text{Asp}$]: functional and molecular modelling studies

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Haemoglobin (Hb) J-Sardegna [$\alpha 50(\text{CE}8)\text{His} \rightarrow \text{Asp}$] is a haemoglobin variant characteristic of subjects from the island of Sardinia. Here we report a study of the functional properties of both fetal and adult Hb J-Sardegna. The results indicate that adult Hb J-Sardegna displays an oxygen affinity that is higher than that of adult Hb only in the presence of 2,3-diphosphoglycerate (2,3-DPG). On the contrary, at 20 °C, the oxygen affinity of fetal Hb J-Sardegna is identical to that of normal fetal haemoglobin, both in the presence and in the absence of 2,3-DPG. A significant difference between these two systems (i.e. a higher oxygen affinity of fetal Hb J-Sardegna) shows up very clearly only when temperature is increased to 37 °C. Hence in

fetal Hb, the main effect of the amino acid substitution is a decrease in the overall enthalpy change of oxygenation. The results outline the role of the α_1 – β_1 interface in assessing the thermodynamics of oxygen binding. The functional properties of both adult and fetal Hb J-Sardegna have been interpreted at the structural level in light of the results obtained by a computational modelling approach performed in comparison with HbA and Hb Aichi, a variant characterized by a different mutation [$\alpha 50(\text{CE}8)\text{His} \rightarrow \text{Arg}$] at the same position.

Key words: Hb variants, $\alpha_1\beta_1$ interface, molecular dynamics, oxygen affinity.

INTRODUCTION

Haemoglobin (Hb) J-Sardegna [$\alpha 50(\text{CE}8)\text{His} \rightarrow \text{Asp}$] was first described in 1968 [1] and better identified in 1989 [2] in subjects from the island of Sardinia. Structural analysis showed an aspartic acid residue in place of the histidine residue at position 50 of the α -globin chain in the region of the polypeptide chain between the C and E helices (CE8). The observed substitution is at the level of an external non-helical segment that, although not directly involved in the haem contacts [3,4], might contribute significantly to maintaining the structure of the haem pocket and therefore to establishing the oxygen-binding properties of the molecule.

The presence of Hb J-Sardegna does not seem to produce any particular clinical effect since appreciable clinical complaints have never been reported in heterozygous carriers of this abnormal Hb [5]. A newborn carrier of this mutation gave us the opportunity to study the functional properties of both fetal and adult Hb J-Sardegna (HbJ), which have never been investigated before. This system, therefore, offers the rare opportunity for characterization of a new Hb variant together with its fetal counterpart.

Of course, since natural Hb variants have been and still are of great help for the elucidation of the critical role played by some amino acid residues, the interest in this system is also linked to the possibility of going deeper into the problem of structure–function interrelationships in a protein molecule. HbJ and its fetal form provide an excellent case for this type of investigation because the substitution of histidine by an aspartic acid residue will result in the loss of the interaction with Glu- $\alpha 30$ of the same

chain, thereby perturbing the interaction of this latter residue with Pro- $\beta 124$ and Pro- $\beta 125$ at the level of the interface between the α_1 and β_1 Hb subunits. Although it has been suggested that this structural region does not play a significant functional role [6], several mutants whose substitution sites are localized at the α_1 – β_1 interface do show a relevant perturbation of the oxygen-binding properties [7]. Moreover, the fetal form gives us the possibility of studying the effect of the observed substitution at the level of a slightly different interface, since β chains are replaced by γ chains.

The functional properties of both HbJ and fetal Hb J-Sardegna (HbFJ) are discussed at the structural level in light of the results obtained by a molecular-modelling study performed in comparison with adult Hb (HbA) and Hb Aichi. This latter, a variant characterized by a different mutation [$\alpha 50(\text{CE}8)\text{His} \rightarrow \text{Arg}$] at the same position, displays a significant degree of instability but normal oxygen-binding properties [8].

MATERIALS AND METHODS

Functional studies

After informed consent, cord blood and venous samples from a newborn and adult heterozygote carriers were collected in an anti-coagulant and treated as usual in order to detect variant Hbs [9,10]. Identification of Hb J-Sardegna was made as described by Manca and Masala [2]. Compositions of the haemolysates were: for adult heterozygote, 75% HbA ($\alpha_2\beta_2$) and 25% HbJ ($\alpha_2^1\beta_2$); and for fetal heterozygote, 60% fetal Hb (HbF; $\alpha_2\gamma_2$), 20% HbFJ ($\alpha_2^1\gamma_2$), 15% HbA ($\alpha_2\beta_2$) and 5% HbJ ($\alpha_2^1\beta_2$). Adult and fetal HbJ present in the respective lysates were purified by FPLC

Abbreviations used: Hb, haemoglobin; HbA, adult haemoglobin; HbF, fetal haemoglobin; HbJ, adult haemoglobin J-Sardegna; HbFJ, fetal haemoglobin J-Sardegna; 2,3-DPG, 2,3-diphosphoglycerate.

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using a HiLoad Q column. The column was first equilibrated with 20 mM Tris/HCl buffer, pH 8.0; then the pH was decreased to 6.1 with a linear gradient. Absorbance was monitored at 280 nm. The adult form of HbJ was obtained in a high degree of purity (HbA was not detectable), whereas HbFJ was contaminated by approximately 10% normal HbF, as checked by electrophoresis [11]. Removal of organic phosphates from Hbs was obtained using a Sephadex G-25 column equilibrated with 0.01 M Tris/HCl buffer, pH 8.0, containing 0.1 M NaCl. Removal of 2,3-diphosphoglycerate (2,3-DPG) has been checked by using a specific analytical kit (665-PA) provided by Sigma Aldrich Fine Chemicals (Milano, Italy). Oxygen-dissociation curves were spectrophotometrically determined by the tonometric method [12] and/or by Hemox-Analyser in the absence and in the presence of the physiological cofactor 2,3-DPG. Spectrophotometric measurements were performed with a Cary 2300 spectrophotometer. A 100-mM stock solution of 2,3-DPG was prepared by dissolving the sodium salt of 2,3-DPG (Sigma) in 0.1 M Tris/HCl buffer plus 0.1 M NaCl, pH 7.0. The overall oxygenation enthalpy (ΔH , kcal/mol; where 1 kcal = 4.184 kJ) was calculated from the van't Hoff equation:

$$\Delta H = -4.574[(T_1 \cdot T_2)/(T_1 - T_2)] \times \Delta \log p50/1000$$

where $p50$ is the partial pressure of the ligand at which 50% of the haems are oxygenated over the temperature range examined (20–30 °C).

Molecular modelling

Molecular-modelling studies were carried out on an INDY R4400 SGI workstation running the IRIX 5.3 operating system, using the software packages InsightII and Discover from MSI (San Diego, CA, U.S.A.). Molecular-modelling studies used X-ray structures as a starting point; for human adult deoxyhaemoglobin the co-ordinates of the 1.9-Å-resolution crystallographic structure [13] were obtained from Brookhaven Protein Data Bank. The structure comprises 574 amino acid residues and 192 crystallographic water molecules. HbJ and Hb Aichi were built from HbA replacing His-50 with Asp and Arg respectively.

Analogously HbFJ was built starting from the 2.5-Å-resolution X-ray structure of fetal human deoxyhaemoglobin [14] of Brookhaven Protein Data Bank. Only one dimer of the HbF crystallographic structure is available, the other was obtained by symmetry.

In the simulations all residues had the conventional protonation states at pH 7. Hence, all Glu, Asp, Arg and Lys residues and the C- and N-termini were fully ionized while the histidine residues were uncharged. Hydrogen-atom co-ordinates were generated using InsightII and all of the hydrogen atoms added to the crystal structures were treated explicitly. Additional water molecules with respect to crystallographic water molecules were added by filling a volume of $3.0 \text{ \AA}^3$ all around the charged residues [15,16]. Solvation was accomplished using the soak command of program InsightII. AMBER All Atoms force field [17,18], as implemented in the MSI software package, was used and a cut-off radius of 15 Å was applied when calculating the non-bonding (van der Waals and Coulomb) interactions. A dielectric constant of 1.0 was used in all simulations. The same procedure was applied to the five structures (HbA, HbJ, HbF, HbFJ and Hb Aichi). First the systems were subjected to energy minimization using the method of steepest descents for 300 iterations with all heavy atoms fixed to remove any artifacts introduced by the addition of explicit hydrogen atoms. Then the protein heavy atoms were fixed and the waters relaxed for 300 iterations. Because of the large number of atoms in the models a

subset was defined by choosing a reference point (the $\alpha 50$ CA, or C α backbone, atom) and constructing a sphere of 15-Å radius around this point. The rest of the system was fixed but included in the calculations of non-bonding interactions. Each entire subset was subjected to minimization using the method of steepest descents for 2000 iterations followed by the method of conjugate gradients (about 5000 iterations) until a root mean square of 0.1 kcal·mol⁻¹·Å⁻¹. Molecular dynamics trajectories of 200 ps were then calculated for each system using the Verlet leapfrog algorithm with a time step of 1 fs [19]. The minimized structures were slowly warmed to 300 K and equilibrated for 20 ps. Following the equilibration procedure, the molecular-dynamics runs were continued for 180 ps at the constant temperature of 300 K. The time-averaged structural properties of each system were evaluated from the final 80 ps of simulation and structural comparisons were made to the respective crystal structures.

RESULTS

Functional studies of HbJ $\alpha_2(\text{His-50} \rightarrow \text{Asp})\beta_2$

The oxygen affinity of purified Hb J-Sardegna was determined in 0.1 M chloride at pH values between 6.8 and 8.0, both in the absence and in the presence of a saturating concentration of 2,3-DPG. The Bohr effect of purified HbJ (which represents about 25% of the total haemolysate), obtained at 20 °C both in the presence and in the absence of 2,3-DPG, is reported in Figure 1

Figure 1 Oxygen Bohr effect of HbA and HbJ

Effect of pH on the oxygen affinity: □, stripped HbA at 20 °C; ■, + 3 mM 2,3-DPG; ●, HbA + 3 mM 2,3-DPG at 37 °C; ◇, stripped HbJ at 20 °C; ◆, + 3 mM 2,3-DPG; ▲, HbJ + 3 mM 2,3-DPG at 37 °C. Conditions: 0.1 M Tris/HCl buffer/0.1 M NaCl. Hb concentration: 3 mg/ml.

Table 1 Overall ΔH of oxygen binding for HbA, HbJ, HbF and HbFJ

The overall ΔH values, in kcal/mol, were calculated from the van't Hoff equation, using the data obtained from oxygen-equilibrium experiments. An average standard deviation of $\pm 15\%$ was obtained.

pH	$-\Delta H_{\text{HbA}}$	$-\Delta H_{\text{HbJ}}$	$-\Delta H_{\text{HbF}}$	$-\Delta H_{\text{HbFJ}}$
7.25	8.4	7.8	6.0	4.1
7.5	10.0	9.2	6.8	5.0

Figure 2 Oxygen Bohr effect of HbF and HbFJ

Effect of pH on the oxygen affinity: $\square$, stripped HbF at 20 °C; $\blacksquare$, +3 mM 2,3-DPG; $\bullet$, HbF + 3 mM 2,3-DPG at 37 °C; $\diamond$, stripped HbFJ at 20 °C; $\blacklozenge$, +3 mM 2,3-DPG; $\blacktriangle$, HbFJ + 3 mM 2,3-DPG at 37 °C. Conditions: 0.1 M Tris/HCl buffer/0.1 M NaCl. Hb concentration: 3 mg/ml.

in comparison with that of normal HbA. In the absence of 2,3-DPG there were no appreciable differences between the functional properties of the two systems. However, upon addition of 2,3-DPG (3 mM) the oxygen affinity of HbJ became significantly higher than that of HbA. Furthermore, since temperature dependence (governed by the associated overall enthalpy change) is another important feature of the reaction of Hb with O_2 , a number of experiments have been performed as a function of temperature within the range of 20–37 °C. The results (see also Figure 1) show that the oxygen-affinity difference between HbJ and HbA observed at 20 °C remained unchanged at 37 °C, indicating that the two systems display the same overall enthalpy change (Table 1).

Finally, as far as homotropic interactions are concerned, it is worthwhile to outline that co-operativity of oxygen binding, as

indicated by the value of the Hill coefficient h_{50} (the slope of the binding curve at 50 % saturation), is not affected by the mutation being very similar if not identical to that displayed by native human Hb ($h_{50} \approx 2.8$) under all the experimental conditions investigated.

Functional studies of HbFJ $\alpha_2(\text{His-50} \rightarrow \text{Asp})\gamma_2$

The oxygen affinity of purified HbFJ was determined in 0.1 M chloride at pH values between 6.5 and 8.0 both in the absence and in the presence of a saturating concentration of 2,3-DPG. The Bohr effect of purified HbFJ (which represents about 20 % of the total haemolysate), obtained at 20 °C both in the presence and in the absence of 2,3-DPG, is reported in Figure 2 in comparison with that of normal HbF. At 20 °C, both in the absence and in the presence of 2,3-DPG, there were no appreciable differences between the functional properties of the two systems. However, at 37 °C (see also Figure 2) and in the presence of 3 mM 2,3-DPG, the oxygen affinity of HbFJ was significantly higher than that of normal HbF, indicating that the two systems are characterized by a different overall enthalpy change (Table 1). Considering the increased oxygen affinity displayed by HbJ, this finding is of particular physiological importance since it contributes to maintaining the difference in oxygen affinity between maternal and fetal blood and therefore the amount of O_2 available for the fetus.

Molecular modelling

Further insight was obtained by analysing the molecular-dynamics simulations of HbA, HbJ, HbF and HbFJ to understand the effect of the replacement of His- $\alpha 50$ with Asp on $\alpha_1\text{--}\beta_1$ and $\alpha_1\text{--}\gamma_1$ interfaces in HbA and HbF respectively. The same approach has been applied to Hb Aichi that is characterized by the substitution of histidine with an arginine residue at the same position. The average structures obtained by the molecular-dynamics simulations may provide a good model for HbJ and HbFJ as well as for Hb Aichi, whose experimentally determined structure is not available. The time evolution of the total potential

Figure 3 Time evolution of total potential energy

The total potential energy (kcal/mol) of the five systems: HbA (thick solid line), HbJ (thin solid line), HbF (thick dashed line), HbFJ (thin dashed line) and Hb Aichi (-x-).

Figure 4 Time evolution of root-mean-square deviation

Root-mean-square deviation (Å) of heavy atoms (solid line) and backbone atoms (broken line) for HbA (a), HbJ (b), HbF (c), HbFJ (d) and Hb Aichi (e).

energy (Figure 3) shows that, after the first ≈ 20 ps of simulations, all trajectories converged, fluctuating about a constant value. In Figure 4 the root-mean-square deviation from the

Figure 5 Comparison of X-ray and molecular-dynamics structures

Superimposition of ribbon diagrams of (A) the X-ray structure of human HbA (red) and the molecular-dynamics average structures of HbA (green), HbJ (violet) and Hb-Aichi (yellow); and (B) the X-ray structure of human HbF (magenta) and the molecular-dynamics average structures of HbF (orange) and HbFJ (blue). Haem is coloured by atom type. Only the $\alpha_1\beta_1$ (or $\alpha_1\gamma_1$) dimer is displayed.

crystal structure is reported as a function of time for both the heavy atoms and the backbones of the five systems. It is seen that the molecular-dynamics structures do not depart significantly from their respective X-ray structures and that the root-mean-square deviations converge to their highest value in the case of HbJ (Figure 4b). The average structures resulting from the last 80 ps of the molecular-dynamics simulations are proposed as a model for the five systems. Figure 5 shows in ribbon format the average structures of HbA, HbJ, Hb-Aichi, HbF and HbFJ

Figure 6 The α_1 - β_1 interface of HbA and HbJ

View of the α_1 - β_1 interface in HbA (a) and HbJ (b). The diagrams include only the environment of the α 50 residue. The models correspond to the molecular-dynamics average structures over the last 80 ps of the simulations. In HbA the hydrogen bond between Glu- α 30 and His- α 50 is displayed as a dotted line. In HbJ the His- α 50 residue is replaced by Asp, which moves towards the solvent and away from the interface.

Figure 7 The α_1 - γ_1 interface of HbF and HbFJ

View of the α_1 - γ_1 interface in HbF (a) and HbFJ (b). In HbF the presence of a Glu residue at γ 125 increases the number of contacts between the two chains with respect to HbA. The hydrogen bond between His- α 50 and Glu- γ 125 is displayed as a dotted line. In HbFJ, as a consequence of the His- α 50 $\rightarrow$ Asp replacement, both the α 50 and the γ 125 residues move towards the solvent and away from the α_1 - γ_1 interface. The Glu- α 30 is no longer bridged by the α 50 residue to the γ 125 amino acid and forms a hydrogen bond with Gly- α 51.

superimposed on their respective X-ray structures. For each model, a view of the environment of the α 50 residue is reported (Figures 6–8). In the case of HbA, the electrostatic interactions between Glu-30 and His-50 (Figure 6a) keep these residues close together and in contact with Pro- β 124 and Pro- β 125. It is worth noting that in HbF Pro- β 125 is replaced by a glutamic acid (in γ chains); as a consequence the specific subunit interface is larger, as evident from Figure 7(a) and Table 2, where the number of contacts involving the α 30 and α 50 residues is reported. A 6-Å cut-off was selected for intersubunit contacts in Hbs [4]. Moreover, as shown in Table 2, the replacement of His- α 50 with Asp leads to a decrease in the number of interface contacts between α_1 and β_1 (or γ_1) chains both in adult and fetal human Hb. In HbJ in particular, Asp-50, interacting negatively with Glu-30, is forced to move towards the outer surface of the protein and the solvent (Figure 6b). The lack of a positive interaction with the

residue at position α 50 gives a high degree of freedom to Glu-30 that results in a significant decrease in the number of contacts with the partner chain.

In Hb Aichi, the situation is completely different because His- α 50 is substituted by an arginine residue that is still able to exchange a positive interaction with Glu-30. Thus as evident from Figure 8, the surface of interaction is even larger than that operative in normal human Hb (see also Table 2).

In HbF, His- α 50 bridges Glu- α 30 and Glu- γ 125 (Figure 7a), increasing the number of contacts with respect to HbA in which Glu- α 30 is involved with the partner chain. Therefore, in HbFJ both α 50 and γ 125 residues move towards the outer surface of the protein and away from the interface (Figure 7b). This leads to a great decrease in the number of contacts.

Figure 8 The α_1 - β_1 interface of Hb Aichi

View of the α_1 - β_1 interface in Hb Aichi. The His- $\alpha 50$ residue is replaced by the basic Arg residue, which interacts positively with the Glu- $\alpha 30$. The number of contacts between the two chains is comparable with that of HbA.

Table 2 Intersubunit contacts for the $\alpha 30$ and $\alpha 50$ residues in HbA, HbF, HbJ, HbFJ and Hb Aichi

Residues at a maximum distance of 6 Å and the number of atoms in this sphere for the $\alpha 30$ and $\alpha 50$ positions are shown. Hydrogen atoms are included.

Residue	HbA		HbF	
	Contacts	No. of atoms	Contacts	No. of atoms
Glu- $\alpha 30$	Pro- $\beta 124$	12	Pro- $\gamma 124$	14
	Pro- $\beta 125$	1	Glu- $\gamma 125$	9
His- $\alpha 50$	Pro- $\beta 124$	8	Pro- $\gamma 124$	8
	Pro- $\beta 125$	3	Glu- $\gamma 125$	13

Residue	HbJ		HbFJ	
	Contacts	No. of atoms	Contacts	No. of atoms
Glu- $\alpha 30$	Pro- $\beta 124$	8	Pro- $\gamma 124$	14
	Ala- $\beta 128$	1	Glu- $\gamma 125$	2
Asp- $\alpha 50$			Ala- $\gamma 128$	1
			Pro- $\gamma 124$	1
			Glu- $\gamma 125$	1

Hb Aichi		
Residue	Contacts	No. of atoms
Glu- $\alpha 30$	Pro- $\beta 124$	11
	Pro- $\beta 125$	1
	Gln- $\beta 127$	1
	Ala- $\beta 128$	1
Arg- $\alpha 50$	Thr- $\beta 123$	3
	Pro- $\beta 124$	11
	Pro- $\beta 125$	1

DISCUSSION

Human HbJ does display an oxygen affinity that is higher than that of HbA. From the data reported in Figure 1 it is clear that, in the absence of 2,3-DPG, the functional properties are identical to those displayed by HbA. The increased oxygen affinity of HbJ has to be ascribed to the lower effect of 2,3-DPG, which appears reduced by about 50 %, both at 20 °C and 37 °C. Hence, the functional alteration is not immediately linked to the structural perturbation induced by the mutation since it shows up only when 2,3-DPG is added to the system. This is at variance with what has been observed on the other abnormal Hb with a mutation at the same position, i.e. Hb Aichi (His- $\alpha 50$ → Arg). Thus this latter Hb, apart from a slight molecular instability (not associated with any clinical disturbances), is characterized by functional properties identical to those of normal human HbA.

This different behaviour finds a rationale on the basis of the computer-modelling analysis reported here. Thus in the case of HbA, the His residue at $\alpha 50$ exchanges electrostatic interactions with the Glu residue at $\alpha 30$ (of the same chain), which in turn is involved in the α_1 - β_1 interface, being in contact with Pro- $\beta 124$ and Pro- $\beta 125$. In Hb Aichi, the arginine residue, which replaces His at $\alpha 50$, is still capable of exchanging a positive interaction with Glu- $\alpha 30$ and the α_1 - β_1 interface is only slightly perturbed. This is in full agreement with the absence of functional effects as reported by Harano et al. [8]. In HbJ the situation is completely different since the Asp residue that substitutes for His at position $\alpha 50$ does interact negatively with Glu- $\alpha 30$, which is forced to move towards the outer surface of the protein. This movement abolishes the interaction with both proline residues of the partner β chain, significantly altering that specific interchain contact.

However, the structural perturbation observed shows up at the functional level only when 2,3-DPG is added to the system. This finding seems of particular significance since it indicates that the α_1 - β_1 interface has a key role in the spreading of the 2,3-DPG effect to the entire molecule. This could be considered of particular interest since it is known that the intradimer α_1 - β_1 interface is essentially characterized by hydrophobic-hydrophobic contacts that are almost not affected by the oxygen-linked conformational transition [20]. Fetal Hbs, due to substitution of β chains with γ chains, add more information and represent a different way of expression of a structural perturbation at the functional level. Thus as evident from Figure 2, at 20 °C the functional properties of HbFJ, both in the absence and in the presence of 2,3-DPG, are identical to those of normal HbF [21,22]. Hence, in spite of the significant structural perturbation induced by the presence of the Asp residue at $\alpha 50$ (see also Figure 7b), the effect of 2,3-DPG appears to be unchanged. This observation could be considered to be a contradiction of the above-reported considerations concerning the case of adult HbJ. However, this contradiction is only apparent when it is considered that HbFs are intrinsically characterized by a significant lower effect of organic phosphates due to the well-known substitutions at the level of the 2,3-DPG-binding site [21,23]. This lower effect could mask that induced by the substitution which, although potentially present, may not show up due to the intrinsic characteristic of the binding site in between the γ chains. The difference between the two fetal systems (HbF and HbFJ) shows up when temperature is increased to 37 °C. Therefore, in this case, the main effect of the structural perturbation induced by the amino acid replacement is the decrease of the overall enthalpy change of oxygenation. This is a quite interesting result since it outlines the role of α_1 - β_1 interface in assessing the thermodynamics of oxygen binding, as already indicated by a recent paper on human (h)/pig (p) hybrid Hbs [24]. In fact, functional

Table 3 HbA, Hb-hybrid and HbF: amino acid differences of β (or γ) chain at the α_1 - β_1 (or α_1 - γ_1) interface

Residue	HbA	Hb-hybrid	HbF
51	Pro	Ala	Ala
112	Cys	Val	Thr
116	His	Arg	Ile
117	His	Arg	His
120	Lys	His	Lys
123	Thr	Asn	Thr
125	Pro	Asp	Glu

Figure 9 Oxygen Bohr effect at 37 °C

Effect of pH on the oxygen affinity of HbA (■), HbJ (◆), HbF (▲) and HbFJ (●) at 37 °C, in the presence of 3 mM 2,3-DPG. Hb concentration: 3 mg/ml.

data indicate that one or more of the substitutions present in the $\alpha_2^h\beta_2^p$ hybrids at the level of the α_1 - β_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 α_1 - β_1 interface in the $\alpha_2^h\beta_2^p$ hybrid compared with human Hb were: Pro- β 51 $\rightarrow$ Ala, Cys- β 112 $\rightarrow$ Val, His- β 116 $\rightarrow$ Arg, His- β 117 $\rightarrow$ Arg, Lys- β 120 $\rightarrow$ His, Thr- β 123 $\rightarrow$ Asn and Pro- β 125 $\rightarrow$ Asp.

By comparison of the amino acid sequences reported in Table 3 we can observe that residues 117, 120 and 123 are the same in human β and γ chains and that Pro- β 125 of HbA is replaced by an acid residue both in fetal and pig Hb (Pro $\rightarrow$ Glu and Pro $\rightarrow$ Asp respectively). Therefore, pig β chain and human γ chain differ only at positions 112 and 116 with respect to the human β chain. On the whole, these abnormal Hbs suggest once more the importance of some (or all) of the positions reported in Table 3 in determining the thermodynamics of oxygen binding.

The physiological significance of this finding appears very clear considering that the fetus affected by the mutation does not suffer any clinical disturbances (i.e. is still able to receive almost the same amount of oxygen from maternal blood) just because the altered thermodynamics of HbF does maintain, with respect to the adult HbJ, the same oxygen-affinity difference that characterizes normal HbF and normal HbA (see Figure 9). This allows oxygen to be transferred from the adult blood to fetal blood in almost the same way, in spite of the increased oxygen affinity displayed by the mutated Hb J-Sardegna.

Finally, we have to consider the hypothesis that the reduced ΔH observed in HbFs may play a key role in the heat exchange from fetus to mother [25]. Therefore it is important to emphasize that the difference in the overall enthalpy change of oxygen binding between adult Hbs and fetal Hbs is almost the same in the 'normal' (HbA/HbF) and the 'abnormal' (HbJ/HbFJ) systems (see Table 1).

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