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Chimeric hemoglobins—Hybrids of human and swine hemoglobin: Assembly and stability of interspecies hybrids

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Abstract

Transgenic swine expressing human HbA contained only one of two types of the anticipated interspecies hybrids, namely $^{H}\alpha_2{}^{P}\beta_2$ (H = human, P = swine). In an attempt to establish whether the absence of the swine α and human β ($^{P}\alpha_2{}^{H}\beta_2$) hybrid in vivo is a reflection of the lack of complementarity between the interspecies chains to generate appropriate interfaces, we have undertaken the in vitro assembly of swine α and human β chimeric tetramer. In contrast to the in vivo transgenic swine system, in vitro the hybrid of swine α human β chain is assembled readily and the hybrid exhibits normal cooperative oxygen binding. Both the swine α human β and the human α swine β interspecies hybrids are stable around neutral pH and do not segregate into parent tetramers even when mixed together. On the other hand, nearly complete exchange of $^{P}\alpha$ chain of $^{P}\alpha_2{}^{H}\beta_2$ hybrid occurs in the presence of $^{H}\alpha$ chain at pH 6.0 and room temperature, resulting in the formation of HbA. However, very little of such an exchange reaction takes place at pH 7.0. These results suggest that the thermodynamic stability of $^{P}\alpha_2{}^{H}\beta_2$ hybrid is lower compared to that of HbA. In contrast, $^{P}\beta$ chain of $^{H}\alpha_2{}^{P}\beta_2$ hybrid is refractory to exchange with $^{H}\beta$ chain at pH 7.0 as well as at pH 6.0, suggesting that the stability of $^{H}\alpha_2{}^{P}\beta_2$ is higher compared to that of HbA ($^{H}\alpha_2{}^{H}\beta_2$). The swine α human β chimeric Hb undergoes subunit exchange reaction with human α-chain in the presence of 0.9 M $MgCl_2$, at pH 7.0. This demonstrates the lower thermodynamic stability of the intradimeric interactions of the heterodimer even at neutral pH. A synergistic coupling of the intra- and interdimeric interactions of the swine α and human β chain heterodimer is essential for the thermodynamic stability of the chimeric Hb under the physiological conditions. Accordingly, we speculate that the lower thermodynamic stability of $^{P}\alpha{}^{H}\beta$ heterodimer (compared to the homodimers $^{H}\alpha{}^{H}\beta$ and $^{P}\alpha{}^{P}\beta$) facilitates its segregation into the homodimers by subunit exchange reaction involving either $^{H}\alpha$ or $^{P}\beta$. This molecular aspect by itself or possibly along with other cellular aspects of the swine system results in the absence of $^{P}\alpha_2{}^{H}\beta_2$ hybrid in transgenic swine expressing HbA.

Keywords: assembly; long-range communication; subunit exchange; synergy; transgenic Hb

The interest in developing murine models for sickle cell disease has resulted in the expression of human HbA and its variants in mice (Behringer et al., 1989; Ryan et al., 1990; Trudel et al., 1991). On the other hand, the generation of transgenic swine expressing HbA is an approach for the production of human pathogen-free hemoglobin on a large scale for the generation of Hb-based oxygen carrier (Swanson et al., 1992; O'Donnell et al., 1993; Rao et al., 1994; Sharma et al., 1994). The expression of human HbA in transgenic animals is anticipated to generate two symmetrical hybrids in addition to the parent hemoglobins. Indeed, when human Hb (or its mutant form) is expressed in mouse, both types of interspecies hybrids are present in the lysates of these transgenic animals (Behringer et al., 1989; Greaves et al., 1990). In contrast, transgenic swine expressing human HbA generate only one of the possible interspecies hybrid, namely, $^{H}\alpha_2{}^{P}\beta_2$, in addition to swine Hb and human HbA (Swanson et al., 1992; O'Donnell et al., 1993).

The assembly of interspecies hybrids is expected to depend on the thermodynamic stability of interfaces, i.e., complementarity between the intersubunit surfaces of the expected new het-

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Abbreviations: HbA, normal human hemoglobin; Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-propane-1,3-diol; IEF, isoelectric focusing.

erodimers. This, in turn, also dictates the propensity of the respective chains to generate the intermediates ($\alpha\beta$ dimers) and their relative concentrations during the assembly of tetramers, and hence should have a profound influence on the kinetics of assembly (Bunn & McDonald, 1983; Bunn, 1987). The generation of interspecies hybrid represents the formation of a new set of $\alpha_1\beta_1$ interface (intradimer) and $\alpha_1\beta_2$ interface (interdimer) as well as the synergy and/or additivity of the interactions of these interfaces. The presence of an interspecies hybrid in a transgenic animal demonstrates the compatibility of the new interfaces and their additivity/synergy.

The observation that transgenic swine contains only $^{H}\alpha_2{}^{P}\beta_2$ hybrid (Swanson et al., 1992) has raised many intriguing questions on assembly of heterodimers or interspecies hybrid tetramers. A simple explanation for the results is the lack of complementarity between the swine α-chain and human β-chain. The corollary of this analysis is that the swine α-chain will fail to generate the interspecies hybrid with human β-chain even in an in vitro assembly system. Such a study will help us to establish whether the lack of swine α human β hybrid in vivo is a simple consequence of the molecular incompatibility of the new interfaces engineered into this modeled hybrid Hb, or other aspects of the tetramer assembly process in the swine system needs to be considered. In an attempt to establish this, we have undertaken the in vitro assembly of the interspecies hybrids containing swine α-chain and human β-chain. The results demonstrate that the absence of the hybrid in vivo is not a direct consequence of the overall thermodynamic stability of the interspecies hybrid. The possible molecular basis for the absence of this interspecies hybrid, in spite of the fact that complementarity exists between the swine α and human β-chains to generate the heterodimer, has been discussed.

Results

Assembly of interspecies hybrid $^{P}\alpha_2{}^{H}\beta_2$ in vitro

The interspecies hybrid of swine α and human β is assembled readily in vitro by the alloplex intermediate pathway. The CM-52 chromatography of the reconstituted material (Fig. 1) shows a major peak that eluted at a position distinct from that of native HbA and swine Hb. The RP-HPLC analysis of the globin chains of this material (Fig. 1, inset A) has confirmed this hybrid as a new species of Hb, a chimeric Hb (interspecies hybrid) containing swine α and human β-chain. The HPLC analysis also clearly reflects the stoichiometry of the swine α and human β chains. The IEF pattern of $^{P}\alpha_2{}^{H}\beta_2$ hybrid also establishes its distinct identity compared to the two parent hemoglobins (Fig. 1, inset B). Thus, although this hybrid is absent in the transgenic swine expressing HbA, this could be assembled readily in vitro by the alloplex intermediate pathway.

Assembly of interspecies hybrid $^{H}\alpha_2{}^{P}\beta_2$ in vitro

The authenticity of the approach, alloplex intermediate pathway, adopted to assemble the $^{P}\alpha_2{}^{H}\beta_2$ hybrid in vitro, has been evaluated by assembling the hybrid $^{H}\alpha_2{}^{P}\beta_2$. This chimeric Hb is present in transgenic swine expressing HbA. The interspecies hybrid assembled in vitro eluted at the same position on CM-cellulose column (chromatogram not shown) as that of the same interspecies hybrid assembled in vivo. The $^{H}\alpha_2{}^{P}\beta_2$ hybrid eluted

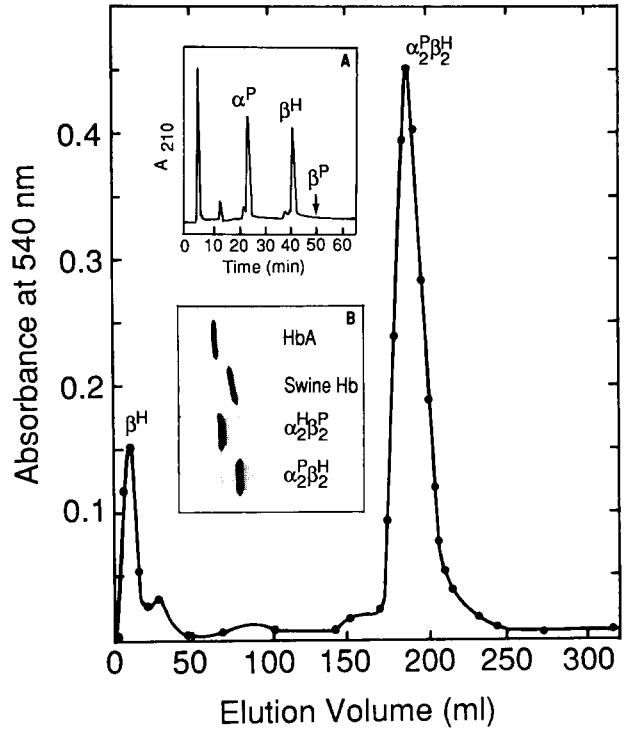

Fig. 1. Purification of $^{P}\alpha_2{}^{H}\beta_2$ interspecies hybrid tetramer by CM-52 chromatography. Reconstituted interspecies hybrid was chromatographed on a CM 52 column (0.9 × 30 cm) employing a linear gradient of 250 mL each of 10 mM potassium phosphate buffer, pH 6.5, and 15 mM potassium phosphate buffer, pH 8.5. The position of the excess β-chain used and the chimeric Hb generated has been identified. Inset A represents separation of globin chains interspecies hybrid on RPHPLC. Vydac C4 column (4.6 × 250 mm) was employed to separate α and β globin chains. Elution of globin chain was monitored at 210 nm. A linear gradient of 35–50% acetonitrile containing 0.1% TFA developed over a period of 100 min was employed to elute globin chains. Flow rate was maintained as 1 mL/mn. Inset B shows the IEF of swine Hb, human HbA, and their interspecies hybrids. Agarose gels with a special blend of pH 6–8 resolve ampholytes were used for isoelectrofocussing studies. Gels were subjected to electrofocusing for 60 min.

from CM-52 cellulose column at the end of the gradient. Its elution position is distinct from the other chimeric Hb, $^{P}\alpha_2{}^{H}\beta_2$, or swine Hb or HbA. The IEF of two interspecies hybrids are also distinct.

Analysis of transgenic swine lysate on CM cellulose

Earlier analysis of lysates of transgenic swine have been performed on DE-52, (Swanson et al., 1992; O'Donnell et al., 1993). As noted above, the electrophoretic mobility of the hybrid $^{P}\alpha_2{}^{H}\beta_2$ is very close to that of swine Hb. It is conceivable that the analytical techniques used in the earlier studies may have failed to detect the presence of $^{P}\alpha_2{}^{H}\beta_2$ hybrid, especially if the hybrid was present only in very small amounts. The resolution of this hybrid on CM-cellulose column from HbA is very good and elutes ahead of swine Hb. Accordingly, the erythrocyte lysates of transgenic swine expressing considerably higher levels of HbA (human HbA at 25% level, the samples analyzed previously had an HbA expression levels of about 10%) has been chromatographed on CM cellulose. Again, besides HbA and

swine Hb, only one additional major new species of Hb is seen in the transgenic swine lysate even when analyzed using the CM-cellulose chromatographic system. The HPLC analysis of this material has confirmed it as a chimera containing human α and swine β-chain. Thus, $^{P}\alpha_2{}^{H}\beta_2$ hybrid is absent even when the expression of HbA in transgenic swine has been increased to 25% and the other hybrid $^{H}\alpha_2{}^{P}\beta_2$ is present at nearly a 30% level.

Oxygen affinity of interspecies hybrids

The functional aspects of the hybrid $^{P}\alpha_2{}^{H}\beta_2$ assembled in the in vitro has been ascertained by studying oxygen-binding parameter (Table 1). The oxygenation behavior of the interspecies hybrids was normal, the Hill coefficients were 2.5 and 2.6 for $^{P}\alpha_2{}^{H}\beta_2$ and $^{H}\alpha_2{}^{P}\beta_2$, respectively. Thus, the hybrids appear to maintain a normal cooperative quaternary structure. The oxygen affinity of swine Hb (P_{50} = 5.8) was intrinsically higher than that of human HbA (P_{50} = 7.2). The interspecies hybrid, $^{H}\alpha_2{}^{P}\beta_2$, is a high oxygen affinity molecule (P_{50} = 5.4) compared to HbA, and its oxygen affinity is indeed closer to that of the swine Hb than to that of HbA. On the other hand, the oxygen affinity of interspecies hybrid $^{P}\alpha_2{}^{H}\beta_2$ is close to that of HbA than to that of swine Hb. Although its affinity appears to be slightly lower (P_{50} = 8.0) than that of HbA (P_{50} = 7.2), the difference in P_{50} values is small enough to unequivocally characterize this hybrid as a low oxygen-affinity species without fully investigating the influence of various allosteric effectors on the oxygen affinity. However, the results certainly demonstrate that the oxygen affinity of interspecies hybrids is closer to that of parent Hb that provided the β-chain than to the one that provided the α-chain.

Modulation of oxygen affinity of chimeric Hbs by DPG

The modulation of oxygen affinity of the hybrids by 2,3 DPG has also been evaluated to establish the influence of the alien α-chain on the interaction of heterotropic effectors as well as to gain further insight into the quaternary structural aspects of the chimeric Hbs (Table 1). The interspecies hybrid, $^{P}\alpha_2{}^{H}\beta_2$, exhibited the lowest sensitivity to 2,3 DPG, and its sensitivity is lower compared to that of either of the parent tetramers. On the other hand, the sensitivity of the oxygen affinity of the interspecies hybrid, $^{H}\alpha_2{}^{P}\beta_2$, is comparable to that of swine Hb. It is of interest to note here that, although the oxygen affinity of the interspecies hybrid, $^{P}\alpha_2{}^{H}\beta_2$, is slightly higher than that of HbA in the presence of DPG, its oxygen affinity is comparable to that of HbA or slightly lower than that of $^{H}\alpha_2{}^{P}\beta_2$ hybrid in the absence of DPG. Interestingly, in the presence of DPG, the swine α human β chimera is the species with the highest oxygen affinity. Accordingly, studies on the influence of buffers and other allosteric effectors should provide new insights into the quaternary structural aspects of this chimeric protein.

Table 1. *Oxygen affinity of chimeric hemoglobins*[a]

Hemoglobin sample	P_{50} −DPG	P_{50} +DPG	Relative increase in P_{50} +DPG/−DPG	Bohr effect
$^{H}\alpha_2{}^{H}\beta_2$	7.2 (2.6)	26	3.6	0.51
$^{P}\alpha_2{}^{P}\beta_2$	5.8 (2.6)	24	3.9	0.29
$^{H}\alpha_2{}^{P}\beta_2$	5.4 (2.6)	21	4.1	0.18
$^{P}\alpha_2{}^{H}\beta_2$	8.0 (2.5)	19	2.4	0.36

[a] All measurements were made at 37 °C in 50 mM Bis-Tris acetate buffer, pH 7.2, at a hemoglobin concentration of 0.5 mM. Values in parenthesis are the *n* values. Values in the presence of DPG were obtained using a twofold molar excess of the effector over the protein. P_{50}, the partial oxygen pressure at half saturation expressed as mm of Hg. Bohr effect was calculated as log P_{50}/pH from the P_{50} values of the proteins at pH 7.0 and 8.0, respectively. The range of error in a single determination of P_{50} values is generally less than 0.5 units. These values are generally determined in triplicates to minimize the errors in these determinations.

Bohr effect of chimeric Hbs

The oxygen affinity of HbA, swine Hb, $^{H}\alpha_2{}^{P}\beta_2$, and $^{P}\alpha_2{}^{H}\beta_2$ hybrids were also determined at different pHs (in the absence of effectors) and the Bohr coefficients have been calculated (Fig. 2; Table 1). The Bohr effect of swine Hb is lower by nearly 40% (Bohr coefficient for Hb = 0.51 and swine Hb = 0.29) compared to that of human HbA under similar conditions. The Bohr effect of $^{P}\alpha_2{}^{H}\beta_2$ hybrid is lower by 25% compared to human HbA (Bohr coefficient for HbA = 0.51; $^{P}\alpha_2{}^{H}\beta_2$ = 0.36). Thus, it is apparent that the swine α-chain contributes about 25% to lower the alkaline Bohr effect of $^{P}\alpha_2{}^{H}\beta_2$ hybrid. The strong contribution of the acid Bohr effect of $^{P}\alpha_2{}^{H}\beta_2$ is seen around pH 6.5, whereas with HbA, it occurs only around pH 5.5. The results demonstrate the distinct quaternary structural aspects of the hybrid relative to the parent proteins. A reverse situation is seen with $^{H}\alpha_2{}^{P}\beta_2$ hybrid. P_{50} value increases considerably in a nonlinear fashion when the pH is lowered from 6.5 to 6.0 (Fig. 4). The alkaline Bohr effect of $^{H}\alpha_2{}^{P}\beta_2$ hybrid is lower by 30% around neutral pH compared to that of swine Hb (Bohr coefficient for swine Hb = 0.29; $^{H}\alpha_2{}^{P}\beta_2$ = 0.18).

First derivative UV spectra of chimeric Hbs

The integrity of the $\alpha_1\beta_2$ interface of the interspecies hybrids has been probed by the first derivative UV spectroscopy. The changes in the spectral properties of Hb around 290 nm has been attributed mainly to the conformational aspects of microenvironment of β37 Trp and β35 Tyr. The former is located at the $\alpha_1\beta_2$ interface of Hb and an insight into the oxy/deoxy conformational transition of this region is reflected in the first derivative spectra of the tetramers. All four proteins exhibited a characteristic sensitivity of their fine structure to the oxy/deoxy conformational transition (data not shown). The derivative spectra of the chimeric proteins were very similar to the parent proteins that provided the β-chains. The oxy/deoxy conformational transition mediated changes reflected in the first derivative spectra of the swine Hb and $^{H}\alpha_2{}^{P}\beta_2$ hybrid were very similar. Similarly, the characteristics of the first derivative spectra of deoxy $^{P}\alpha_2{}^{H}\beta_2$ hybrid was closer to that of deoxy HbA, although not identical to that of deoxy HbA. Therefore, the perturbation of $\alpha_1\beta_2$ interface of $^{P}\alpha_2{}^{H}\beta_2$ hybrid, if present, is very minimal and overall conformational aspects of chimeric Hb appear to be comparable to that of HbA.

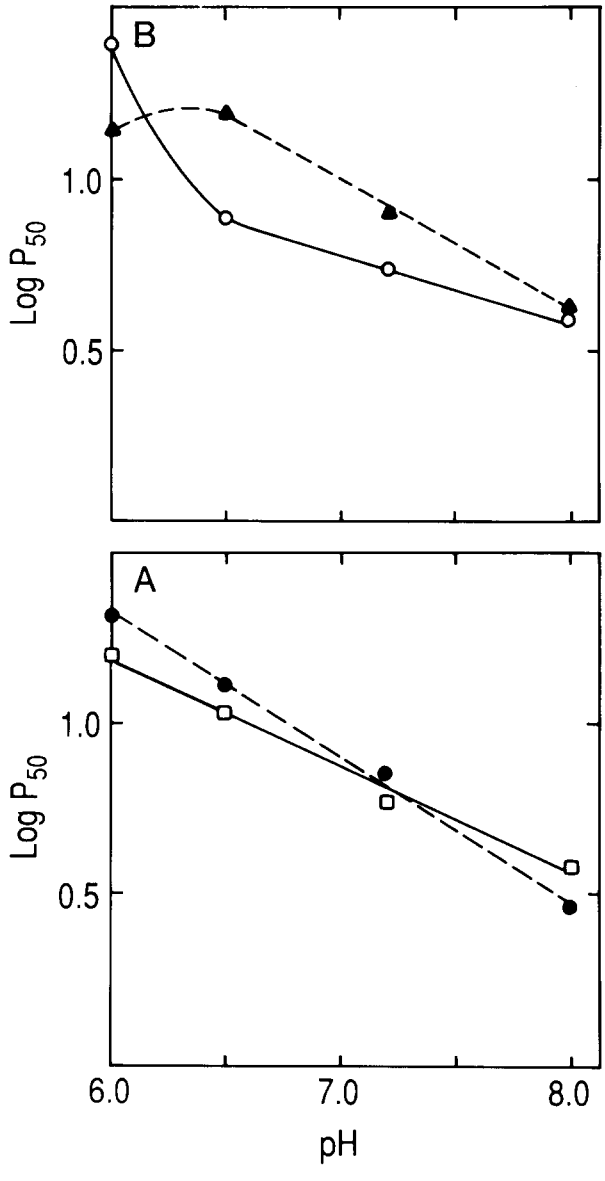

Fig. 2. Bohr effect of HbA, swine Hb, and interspecies hybrids. Oxygen equilibrium curves were recorded using Heme-O-Scan in 50 mM Bis-Tris acetate buffer and at 37 °C and the protein concentration was maintained as 0.5 mM on a tetramer basis for all the studies. The range of error in a single determination of P_{50} value is generally less than 0.5 units. These values are generally determined in triplicates to minimize the errors in these determinations. **A:** Data for human HbA (filled circles) and swine Hb (open squares). **B:** Data for the interspecies hybrids ${}^{P}\alpha_2{}^{H}\beta_2$ (filled triangles) and ${}^{H}\alpha_2{}^{P}\beta_2$ (open circles).

Stability of the chimeric Hbs

In an attempt to gain further insight into the molecular aspects of chimeric Hbs, particularly about the overall thermodynamic stability of the tetramer, the susceptibility of the chimeric Hbs to proteolytic digestion has been investigated. HbA is resistant to V8 protease digestion, whereas the isolated α- and β-chains are digested readily by V8 protease (Seetharam et al., 1986). The ${}^{P}\alpha_2{}^{H}\beta_2$ hybrid exhibited a complete resistance to V8 proteolytic digestion, just as seen with human HbA (chromatogram not shown). This reflects that the normal Hb fold is conserved in ${}^{P}\alpha_2{}^{H}\beta_2$ hybrid. Similar resistance to proteolysis is seen with swine Hb and human α swine β chimeric Hb as well.

The integrity of the quaternary structure of chimeric Hbs has been analyzed by FPLC analysis of the samples on a Superose 12 column using phosphate buffered saline, pH 7.4, as the eluant. If the $\alpha_1\beta_2$ interface of the chimeric Hbs are not as stable as the parent Hbs, dissociation of the tetramer into $\alpha\beta$ dimer could occur under the physiological conditions. Such a dissociation will be apparent in the gel filtration behavior of the sample. The elution patterns of the chimeric Hbs and the parent species are indistinguishable from one another (all species elute at an elution volume of 15.5 mL; data not shown). Gel filtration pattern of the chimeric Hb did not show any signs of the dissociation of the ${}^{P}\alpha_2{}^{H}\beta_2$ chimera. The results confirm the generation of interface interactions at the respective $\alpha_1\beta_2$ interfaces that are strong enough to assemble stable tetrameric structures of swine α and human β-chains with an apparent molecular size of 64,000 Da.

The stability of the intradimeric interactions in the heterodimer (interactions of $\alpha_1\beta_1$ interface) of swine α and human β chains has also been probed by subjecting the chimeric Hbs to molecular sieve chromatography on Superose 12 column using 0.9 M $MgCl_2$, pH 7.0, as the eluant (Benesch & Kwong, 1991). In the presence of 0.9 M $MgCl_2$, the swine as well as human Hb tetramers dissociate quantitatively into respective $\alpha\beta$ dimers and elute at an elution volume of 16.8 mL (Fig. 3A). The swine α human β chimeric Hb also dissociated into $\alpha\beta$ dimer just as the parent Hbs and elute at an elution volume of 16.8 mL (Fig. 3B). Apparently, the dissociation of the $\alpha\beta$ heterodimer into monomers did not occur even in the presence of 0.9 M $MgCl_2$, because the dissociated monomers elute at an elution volume of 17.9 mL (Fig. 3C). If the $\alpha_1\beta_1$ interface of the heterodimer are not compatible to generate stable interface, one could anticipate the dissociation of the heterodimers into the chains (monomeric subunits). The gel filtration results suggest that the complimentation present between the swine α and human β chains is strong enough to permit the formation of ${}^{P}\alpha{}^{H}\beta$ heterodimer under the physiological conditions (Fig. 3).

Relative thermodynamic stability of chimeric Hbs—Subunit exchange assay

Even though the complimentarity between the swine α chain and human β-chain is sufficient for them to exist as $\alpha\beta$ heterodimers, the thermodynamic stability of the intersubunit interactions in the heterodimers may not be as strong as in the homodimers. Accordingly, a subunit exchange assay has been developed to probe the relative thermodynamic stability of the chimeric Hbs. This assay involves the incubation of the hybrids with the appropriate subunits to facilitate the segregation of the heterodimers into homodimers, if such a segregation process is thermodynamically feasible. The exchange of subunits occur only if the thermodynamic stability of the homodimer is higher than that of the heterodimer and only when the heterodimers are in equilibrium with their monomers. The chimeric Hbs are incubated with the respective subunits or tetramers for desired period (up to 24 h) at room temperature. These samples are then subjected to IEF to establish whether these chimeric Hbs have segregated into the respective parent tetramers. Initially, the two chimeric Hbs were incubated together at pH 7.0. The mixture of the chimeric Hbs failed to segregate into the parent proteins. Thus, the

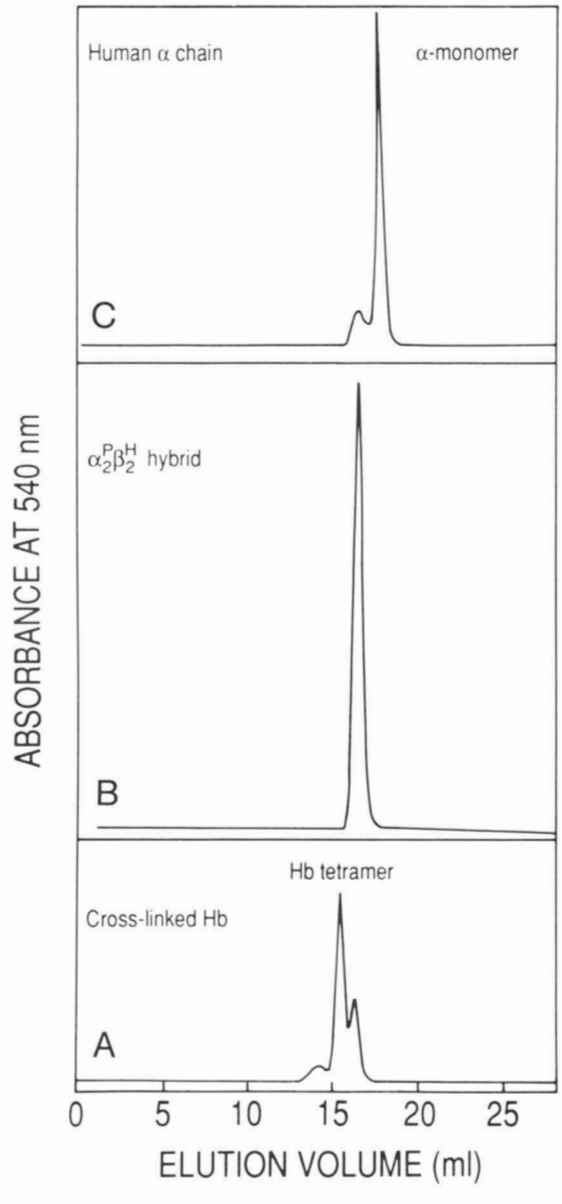

Fig. 3. Gel filtration of HbA, $^P\alpha_2{}^H\beta_2$ and crosslinked HbA on Superose-12 column. Proteins were gel filtered on FPLC using Superose-12 column and 0.9 M $MgCl_2$ in 50 mM Bis-Tris, pH 7.4, buffer was used as eluent. Elution of the proteins was monitored at 540 nm.

absence of the chimeric Hb containing swine α-chain and human β-chin in vivo is not a reflection of differential stability of chimeric Hbs compared to the parent species under the physiological conditions once they are formed.

The absence of segregation of the hybrid species into a parent tetramer at neutral pH could also be a consequence of the stability of one of the heterodimers, which restricts the dissociation of the heterodimer into chains, the species needed to initiate the segregation process. But, if the interspecies hybrid is mixed with either the $^H\alpha$ or $^H\beta$ chains (complimentary to the $^H\alpha$ or $^H\beta$ chains present in the hybrid, respectively), the heterodimer with lower stability will have the opportunity to exchange their subunits to form the respective native tetramer (HbA). Nonetheless, even when $^P\alpha_2{}^H\beta_2$ hybrid is incubated with the isolated human α-chain at pH 7.0, the segregation of the chimeric Hb into native tetramer did not occur.

Influence of pH on the subunit exchange

The sharp transition of the pH–oxygen affinity correlation curve (Fig. 2) of the chimeric Hb containing swine α and human β chains around pH 6.5 is a reflection of some structural transition of the hybrids in this pH region. The hybrids could reflect maximum structural/conformational differences around this pH compared to its parent molecule. Accordingly, the subunit exchange experiments have also been conducted at pH 6.0. In contrast to the results obtained at the neutral pH, the $^P\alpha_2{}^H\beta_2$ hybrid slowly exchanged its swine α-chain with the human α-chain to generate HbA (Fig. 4A, top panel). However, the exchange was very slow, and the complete exchange took nearly 24 h even with a fourfold molar excess of human α-chain (bottom panel). In contrast, the other hybrid, human α swine β hy-

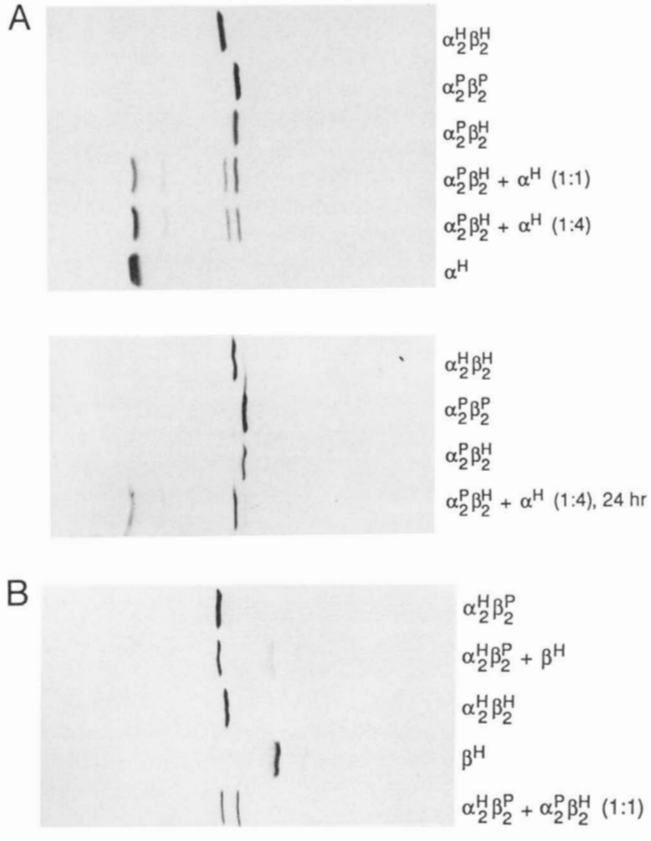

Fig. 4. Subunit exchange with chimeric Hbs. IEF of swine and human hemoglobin interspecies hybrids subjected to exchange reaction for 24 h. After incubation, proteins were applied onto agarose IEF gels with a blend of pH 6–8 resolve ampholytes. **A:** Top panel. Lane 1, HbA; lane 2, swine Hb; lane 3, $^P\alpha_2{}^H\beta_2$; lane 4, $^P\alpha_2{}^H\beta_2 + {}^H\alpha$ (equimolar amounts of the α-chain and the chimeric Hb; lane 5, $^P\alpha_2{}^H\beta_2 + {}^H\alpha$ (the human α-chain was present in fourfold molar excess over the chimeric Hb); and lane 6, $^H\alpha$;. With these samples, the exchange reaction was carried out for 3 h. Bottom panel shows the pattern after 24 h of the incubation. Figure 4B demonstrates the stability of the $\alpha_1\beta_1$ interface of the human α swine β chimera as reflected in the subunit exchange assay. Lane 1, $^H\alpha_2{}^P\beta_2 + {}^H\beta$; lane 2, HbA ($^H\alpha_2{}^H\beta_2$); lane 3, $^H\beta$; lane 4, $^P\alpha_2{}^H\beta_2 + {}^H\alpha_2{}^P\beta_2$.

brid, did not exchange its β-chain with human β-chain (Fig. 4B). Thus, it is clear that the thermodynamic stability of the $^{P}\alpha_2{}^{H}\beta_2$ hybrid is lower than that of $^{H}\alpha_2{}^{P}\beta_2$ hybrid and that of $^{H}\alpha_2{}^{P}\beta_2$ is higher than that of $^{H}\alpha_2{}^{H}\beta_2$. The subunit exchange seen with $^{P}\alpha_2{}^{H}\beta_2$ hybrid at pH 6.0 is a reflection of the lower thermodynamic stability of the $\alpha_1\beta_1$ interface of $^{P}\alpha_2{}^{H}\beta_2$ hybrid. The dissociation of the αβ heterodimer ($^{P}\alpha{}^{H}\beta$) into swine α human β chain is the rate-limiting step for this segregation reaction. However, the mixture of two chimeric Hbs by themselves did not segregate into parent tetramers even at pH 6.0, reflecting the stability of the chimeric Hb under the physiological conditions once they are formed.

Destabilization of the $\alpha_1\beta_1$ interface of the heterodimer occurs on lowering the pH from the neutral region (pH 7.0) to the slightly acidic region (pH 6.0), and this leads to the subunit exchange. However, under physiological pH (neutral), the intrinsic thermodynamic stability of this interface is strong enough and prevents the subunit exchange reaction. Alternately, the lack of the subunit exchange reaction with the $^{P}\alpha_2{}^{H}\beta_2$ chimera at pH of 7.0 is a reflection of the stability afforded by the interactions of the $\alpha_1\beta_2$ interface to the $\alpha_1\beta_1$ interface of the chimera. Therefore, if the interactions of the $\alpha_1\beta_2$ interface are to be weakened at neutral pH as to generate αβ heterodimer, the subunit exchange reaction will take place if the intrinsic thermodynamic stability of the $\alpha_1\beta_1$ interface of the heterodimer is lower compared to the homodimer even at the neutral pH. Accordingly, the subunit exchange experiments have been conducted with the chimeric Hb in the presence of 0.9 M $MgCl_2$, pH 7.4 (condition known to generate αβ dimer under the physiological pH [Benesch & Kwong, 1991]). These conditions weaken the $\alpha_1\beta_2$ interface interactions of the tetramer, and results in the quantitative conversion of the tetramer into αβ dimers. The heterodimer of $^{P}\alpha_2{}^{H}\beta_2$ chimera exchanged its α-chain with the human α-chain at pH 7.0 to generate the homodimer $^{H}\alpha{}^{H}\beta$ in the presence of 0.9 M $MgCl_2$, and when the $MgCl_2$ is dialyzed off these homodimers generated human HbA. As demonstrated earlier (Fig. 3), the intradimeric interactions of the swine α human β chimera are strong enough to exist as $^{P}\alpha{}^{H}\beta$ heterodimer in the presence of 0.9 M $MgCl_2$ and the heterodimer does not dissociate into monomeric α and β chains (in the presence of 0.9 M $MgCl_2$). Therefore, the generation of homodimer from the chimeric dimer (heterodimer) is truly a subunit exchange phenomenon. The generation of HbA does not represent a recombination of the fully dissociated human β-chain with the human α-chain added to the system. The subunit exchange at neutral pH (in the presence of $MgCl_2$) demonstrates that, under the physiological conditions, the interheterodimer interactions compensate, at least to a large degree, the weaker the intraheterodimer interactions and facilitate the conservation of the quaternary structure of the chimeric Hb under the physiological conditions. Accordingly, at neutral pH, no subunit exchange reaction is observed in the absence of $MgCl_2$.

Stability of the chimeric Hb, $^{H}\alpha_2{}^{P}\beta_2$, in the presence of $^{H}\beta$ chains

The segregation of chimeric Hb containing swine α-chain in vitro suggests that the thermodynamic stability of the $\alpha_1\beta_1$ interface of this interspecies hybrid is lower compared to the one present in the parent Hb, namely HbA. In an attempt to establish whether this is true also of the other chimeric Hb, namely the one containing the β-chain from the swine, the propensity of this hybrid to segregate into HbA at pH 7.0 containing 0.9 M $MgCl_2$ and human β-chain has also been pursued. This hybrid is stable even in the presence of the β-chain from human, reflecting a higher thermodynamic stability of this heterodimer compared to the parent HbA (Fig. 4B). Therefore, it is suggested that the thermodynamic stability of the tetramers increases, in the order, chimeric Hb with swine α-chain, HbA, chimeric Hb with swine β-chain, and swine Hb.

Discussion

The in vitro assembly of chimeric hemoglobins of HbA and swine Hb by the alloplex intermediate pathway establishes that the absence of the $^{P}\alpha_2{}^{H}\beta_2$ interspecies hybrid in transgenic swine expressing human HbA is not a consequence of lack of complementarity between the human β-chain and the swine α-chain. The interspecies hybrid generated in vitro exhibits normal cooperative oxygen-binding properties that are characteristic of the tetrameric Hb. The oxygen affinity of $^{P}\alpha_2{}^{H}\beta_2$ hybrid, is close to that of human HbA. Similarly, the oxygen affinity of the $^{H}\alpha_2{}^{P}\beta_2$ hybrid is close to that of the swine Hb. Thus, the oxygen affinity of the interspecies chimeric Hbs appears to be dictated predominantly by the β chain. Therefore, the conclusion is that the swine and human α-chains are nearly equivalent in generating a functional cooperative tetrameric structure.

However, this is not the complete picture. The interspecies hybrid appears to have native-like quaternary structural features as reflected by the modulation of the oxygen affinity in the presence of allosteric effector, 2,3-DPG (binds to ββ cleft). However, it should be noted that the DPG binding pocket of Hb is generated primarily by the interactions of the two β-chains. Accordingly, the differential modulation of the oxygen affinity of the hybrids (relative to the parent Hbs) by DPG can be considered as a reflection of the integrity of the conformational aspects of ββ cleft in the hybrid relative to its parent tetramer, which has been influenced by the primary structure and the consequent conformational aspects of α-chain. Although the oxygen affinity of both the parent and chimeric Hbs are sensitive to the presence of DPG, the sensitivity of the hybrids is distinct compared to the parent tetramer that provided the β-chain. Both HbA and swine Hb exhibit a relative sensitivity to DPG that is nearly the same even though there are 44 sequence differences (per αβ dimer) in swine Hb compared to human HbA. The $^{P}\alpha$-chain reduces (compared to $^{H}\alpha$) the relative sensitivity of the tetramer to the presence of DPG (to reduce the oxygen affinity) when it is hybridized with $^{H}\beta$. On the contrary, the influence of the $^{H}\alpha$ chain in the tetramer containing $^{P}\beta$ on the modulation of the intrinsic oxygen affinity of the molecule by DPG is nearly comparable to that of the $^{P}\alpha$ in swine Hb. The amino acid sequence of the regions of β-chains of human and swine that participate in the formation of ββ cleft is conserved. Thus, the overall structure of DPG pocket of chimeric Hbs is expected to be comparable to that in the native tetramers containing the respective β-chains. Therefore, the differential response of the chimeric Hbs to DPG reflects the communication of the conformational consequence of the sequence differences of the alien α-chain of the chimeric Hb to their ββ cleft, namely a long-range communication of the structural perturbation of αα or αβ interaction to the ββ cleft of the chimeric Hb molecule.

The distinctive structural features of the chimeric Hbs relative to the parent tetramers is also reflected in their Bohr effect. The Bohr coefficient of swine Hb is nearly 40% lower compared to that of human HbA in 50 mM Bis-Tris acetate buffer at pH 7.0 and 37 °C. Similar observations have been made by Sinet et al. (1982), and suggested that the decreased Bohr effect may be due to decreased interactions of the Val-1(α) and Asn-131(α). Recently, Katz et al. (1994) suggested that A helix of α-subunit shifts away from the protein core by about 0.5 Å and a hydrogen bond exists between Val-1(α2) and Asn-131(α2). This hydrogen bond does not exist in human HbA. The amino acid sequence differences also provide clues for the identification of the Bohr groups perturbed in the swine Hb. Ho and his colleagues (Sun et al., 1994) have demonstrated recently the contribution of His-20(α) to the alkaline Bohr effect of Hb. The mutation of His-20(α) to Gln results in 25–30% reduction in the alkaline Bohr effect. In swine α-chain, His-20(α) is mutated to Gln. It is conceivable that at least a part of the lowered Bohr effect in swine Hb is a consequence of the mutation of His-20(α) to Gln. Consistent with this, the Bohr effect of $^{P}\alpha_2{}^{H}\beta_2$ is also nearly 25% lower than that of HbA ($^{H}\alpha_2{}^{H}\beta_2$). However, the other chimeric Hb, $^{H}\alpha_2{}^{P}\beta_2$, does not behave like HbA, reflecting the contribution of other structural elements of swine Hb in dictating its Bohr effect. The study suggests the contribution of structural elements of swine β-chain in dictating the overall differences in the Bohr effect of swine and human HbA.

Another interesting aspect that emerges from the present study of chimeric Hbs is that, in the parent Hbs, a unique balancing of contributions of the acid and alkaline Bohr groups of α and the β-chains exists. Both α-chains and β-chains of swine Hb contribute a mutually compensating influence to conserve the linear inverse correlation between the P_{50} and the proton concentration up to at least pH 6.0. In contrast, in the $^{P}\alpha_2{}^{H}\beta_2$ chimera, the contribution of the acid Bohr groups become more pronounced below pH 6.5 compared to the parent HbA itself. Presumably, this is a consequence of the absence of His-20(α) in the swine α-chain. However, when this Bohr group [His-20(α) of human α-chain] is present, the $^{H}\alpha_2{}^{P}\beta_2$ chimera, the onset of acid Bohr effect is pushed to a much lower pH compared to swine α human β chimeric Hb. Thus, the role of the β-chains in mutually compensating the contributions (or lack of contribution) of α-chain is apparent in the swine and human Hbs. A further insight into the molecular basis of the Bohr effects in the chimeric Hb should help in the design of Hb-based oxygen carriers, the oxygen affinity of which exhibit unusual sensitivity to low pH (compared to human HbA).

The success in assembling the chimeric Hb in vitro should be considered as the demonstration of the compatibility of intersubunit surfaces to generate a tetrameric structure. In structural terms, this implies the potential to generate a set of new $\alpha_1\beta_1$ and $\alpha_1\beta_2$ interfaces within the constraints of the basic hemoglobin fold. The formation of the chimeric Hb in vivo should be dictated by the relative thermodynamic stability of the various combinations of the subunits possible. The subunit exchange reaction developed in the present study reflects the perturbations of the energetic aspects of the newly engineered intersubunit surfaces of the heterodimers, namely, $^{P}\alpha{}^{H}\beta$ and $^{H}\alpha{}^{P}\beta$. The fact that, at pH 6.0, the segregation of the $^{P}\alpha_2{}^{H}\beta_2$ hybrid into HbA occurs in the presence of human α-chain demonstrates that the $\alpha_1\beta_1$ interface of the $^{P}\alpha{}^{H}\beta$ dimer (heterodimer) has a lower thermodynamic stability compared to the homodimer $^{P}\alpha{}^{P}\beta$ or $^{H}\alpha{}^{H}\beta$. The lower thermodynamic stability of the intradimeric interfaces is true even at neutral pH.

The exchange of $^{P}\alpha$ chain of the heterodimer by the human α-chain reflects a weaker $\alpha_1\beta_1$ interface in the heterodimer of swine α and human β-chains. The lack of segregation when chimeric tetramers are mixed together suggests that the integration of the interaction of $\alpha_1\beta_1$ and $\alpha_1\beta_2$ interfaces in the swine α human β chimeric Hb compensates for the intrinsic lower thermodynamic stability of $\alpha_1\beta_1$ interface of the $^{P}\alpha{}^{H}\beta$ heterodimer detected at pH 6.0. The $\alpha_1\beta_2$ interface interactions in $^{P}\alpha_2{}^{H}\beta_2$ hybrid, either by itself or coupled with the interactions of the central cavity (including the interactions of the DPG binding pocket), adds significantly to the stabilization of the $\alpha_1\beta_1$ interface of the heterodimer and endows the stability to the tetrameric structure. Thus, unique communication pathways of stabilization of intersubunit surface is operational in the interspecies hybrid.

The coupling of the noncovalent interactions of these newly engineered interfaces (communication pathways) are very sensitive to pH. The conditions that favor subunit exchange in the hybrids, (i.e., pH 6.0) exhibit a higher influence on the overall tetrameric structure in the case of the hybrids than in the case of HbA. These studies support the concept that the stability of the assembled tetramer represents not simply the net sum of the stabilization energies of various subunit interfaces; it represents the synergy of the interactions of the intersubunit surfaces. The lower thermodynamic stability of one interface could be compensated by the synergy of the stabilization energetics of the individual interfaces. Such a diversity or a flexibility in molecular aspects for stabilization of cooperative tetrameric structure of Hb should have played a pivotal role in the conservation of the basic Hb fold during evolution.

The demonstration of the lower thermodynamic stability of the swine α human β heterodimers leads to a hypothesis that the heterodimer generated during the assembly in vivo could segregate readily to form homodimer if the appropriate partner is available to exchange. However, once the heterodimers are stabilized as tetramers by the formation of $\alpha_1\beta_2$ interface, as achieved during the in vitro assembly of the chimera (tetrameric structure), very little segregation takes place under the physiological conditions.

The intriguing question now is the molecular basis for the lower thermodynamic stability of swine α human β heterodimers (compared to human $\alpha\beta$ homodimer) and the higher thermodynamic stability of the human α swine β heterodimer. Both of the chimeric Hbs can be assembled in vitro, but only $^{P}\alpha_2{}^{H}\beta_2$ is assembled in vivo. However, the transgenic mice expressing Hb assembled both types of chimeric hemoglobins in vivo. The α-chains of mouse and swine differ from the human α-chain with 19 and 22 sequence differences (Braunitzer et al., 1978), respectively. Of these, seven sequence differences are common between mouse α-chain and swine α-chain, and these are at α^{19}, α^{71}, α^{73}, α^{76}, α^{78}, α^{113}, and α^{116}. One or more of the remaining 13 sequence differences of swine α-chain has contributed to lack of chimeric Hb containing the swine α-chain and human β-chains. Of all the remaining sequence differences, it is of interest to note that two of the sequence differences of swine α-chain are indeed located at the $\alpha_1\beta_1$ interface contact sites. These sequence differences are α35 Ser → Gly and α118 Thr → Asn (Fig. 5). Interestingly, both of these sequence differences are present only in swine α-chain and not in mouse α-chain.

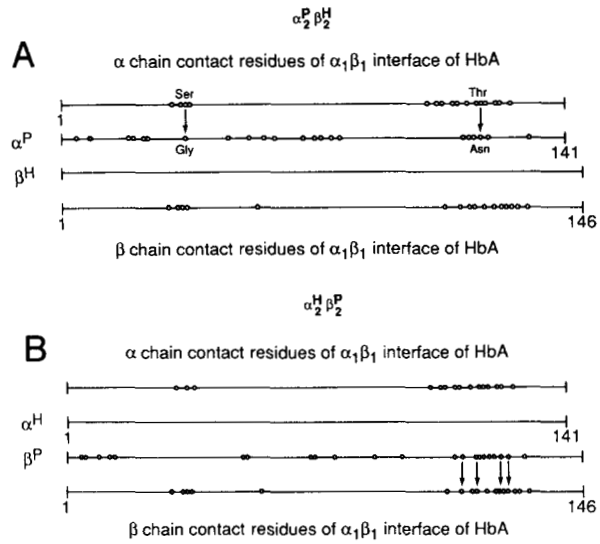

Fig. 5. Schematic representation of the sequence differences located at the $\alpha_1\beta_1$ interfaces of the chimeric Hb, $^P\alpha_2{}^H\beta_2$ and $^H\alpha_2{}^P\beta_2$. The $\alpha_1\beta_1$ interface residues in the human Hb depict the region of the polypeptide chain involved in forming this hybrid. The sites in the $^P\alpha$ and $^P\beta$ chains of the respective chimeric hemoglobins where a sequence difference is located at the interface is indicated by an arrow pointing to the respective chains. **A:** Chimeric Hb containing the α-chain from swine. **B:** Chimeric Hb containing β-chain from swine.

In the case of the chimeric Hb, $^H\alpha_2{}^P\beta_2$ hybrid, swine β-chain contains 22 sequence differences compared to human β-chain. Of these, four differences are located at $\alpha_1\beta_1$ interface, i.e., β112 Cys → Val, β116 His → Arg, β122 Phe → Asn and β125 Pro → Asp (Fig. 5B). Presumably, these sequence differences are responsible for the higher stability of $^H\alpha^P\beta$ heterodimer and also for the refractory nature of $^H\alpha_2{}^P\beta_2$ hybrid to exchange its subunit. It is of interest to note here that the mutation of β 112 Cys → Gly has been implicated in the stabilization of the $\alpha_1\beta_1$ interface (Fronticelli et al., 1994). Similarly, expression of HbA with a single amino acid substitution at β112 Cys → Val in transgenic mice (White et al., 1994) influenced the relative ratios of the interspecies hybrids (compared to the mice expressing the normal HbA).

The lower thermodynamic stability of the heterodimer, $^P\alpha^H\beta$, by itself may not be the sole contributor to the total absence of the chimeric Hb, $^P\alpha_2{}^H\beta_2$, in vivo. The other physiological factors could further modulate this biophysical phenomenon. The concentration of the heterodimers in vivo will be dictated by their thermodynamic stability on one hand, and the concentration of the two chains on the other. The apparent higher thermodynamic stability of the heterodimer, $^H\alpha^P\beta$, is expected to keep the relative concentration of the free $^P\beta$ low in the system. Accordingly, $^H\beta$ chain will be trapped by the free $^H\alpha$-chains, thereby limiting the concentration of $^H\beta$ chains for the formation of the heterodimer, $^P\alpha^H\beta$. Even if some amount should be formed transiently, these could segregate readily as $^P\alpha^P\beta$ dimer. Besides these thermodynamic factors, other factors, such as the amount of $^H\alpha$ expressed in swine, may be expected to influence the amount of the two tetramers containing $^H\alpha$ formed, namely $^H\alpha_2{}^H\beta_2$ and $^H\alpha_2{}^P\beta_2$. The relative ratio of $^H\alpha_2{}^P\beta_2$ to $^H\alpha_2{}^H\beta_2$ is also varied considerably between the transgenic swine. If the thermodynamic stability of the heterodimers is the only factor that determines the relative distribution of $^H\alpha$ transcribed in the swine system as $^H\alpha_2{}^H\beta_2$ and $^H\alpha_2{}^P\beta_2$, the relative distribution of the tetramers containing $^H\alpha$ should remain constant between the transgenic swine. However, this is not the case. Thus, other factors appear to be contributing to the distribution of the alien chains into tetramers (Bunn, 1987; Bunn & McDonough, 1974; Bunn & McDonald, 1983; Shaeffer et al., 1984). Possible contribution of the thermodynamic stability of the heterodimer in the kinetics of the assembly of $^P\alpha^H\beta$ dimer is also not apparent from the present study. The tetramer is assembled in the present study by the alloplex intermediate pathway. Isolation of heme containing swine α and β globin chains and the assembly of the heterodimers will be needed before the studies are focused on the possible role of cellular factors, such as chaperonins, in the assembly of Hb tetramers in vivo. Nonetheless, based on the results of the present study, we suggest that the absence of swine α human β hybrid in vivo is a synergistic consequence of the lower thermodynamic stability of the $^P\alpha^H\beta$ heterodimer, higher thermodynamic stability of heterodimer, $^H\alpha^P\beta$, and the limiting amount of $^H\beta$ chain expressed in vivo. Future crystallographic and NMR investigations of the intersubunit interfaces of the chimeric Hbs of the type discussed here and assembled previously by us (Roy et al., 1995) should help us to delineate the communication pathways of the structural information between intersubunit interfaces; particularly from the *trans* α-chains to the ββ interfaces.

Materials and methods

Preparation of the α and β chains of HbA and swine Hb

Erythrocytes from human and transgenic swine were used to prepare the hemolysates and lysates were dialyzed extensively against phosphate buffer with saline, pH 7.4. Stripped lysates were purified by DEAE and CM 52 chromatography. Purified HbA was reacted with HMB (*p*-hydroxymercuribenzoate) to isolate α and β globin chains as described by Bucci (1981). Swine Hb chains were prepared by CM 52 chromatography of acid-acetone precipitated globin in the presence of 8 M urea (Clegg et al., 1966; Roy & Acharya, 1994). The acid-acetone precipitated total globin of swine was dialyzed against 50 volumes of starting buffer (5 mM disodium phosphate, 8 M urea and 50 mM β-mercaptoethanol, the pH of the buffer was adjusted with dilute phosphoric acid after addition of urea and mercaptoethanol to 6.85) for 2–3 h. After dialysis, globin was loaded onto CM 52 column (0.4 × 18 cm) that was pre-equilibrated with starting buffer. The globin concentration was maintained at 8 mg/mL during loading the globin onto the column. Separation of α and β globins was performed using a linear gradient of 200 mL, each of starting buffer (5 mM disodium phosphate buffer containing 8 M urea and 50 mM β-mercaptoethanol) and 30 mM disodium phosphate buffer, pH 6.85, containing 8 M urea and 50 mM β-mercaptoethanol. Elution of globin chains was monitored at 280 nm. The β globin eluted first from the column and α globin was eluted at the end of the gradient, thus providing a clear cut separation between the two globin chains. Fractions containing globins were pooled separately and dialyzed extensively against 0.1% acetic acid. Dialyzed globin chains were lyophilized and stored at −80 °C until use.

Preparation of interspecies hybrids of swine-human hemoglobins

The assembly of the interspecies hybrids using globin chains of swine Hb and the heme containing complimentary human Hb chains was carried out by the alloplex pathway as discussed previously (Roy & Acharya, 1994; Roy et al., 1995).

Purification and characterization of interspecies hybrids

The reconstituted and reduced interspecies hybrids were dialyzed with 10 mM phosphate buffer, pH 6.5, and chromatographed on a CM-52 column (0.9 × 30 cm) equilibrated with 10 mM potassium phosphate buffer, pH 6.5, containing 1 mM EDTA. The protein was eluted using a linear pH and ionic strength gradient generated by using 250 mL each of 10 mM potassium phosphate buffer, pH 6.5, and 15 mM potassium phosphate buffer, pH 8.5, respectively, as the initial and final buffers. Both buffers contained 1 mM EDTA. Elution of proteins from column was monitored at 540 nm. The identification of a particular species eluting from the CM-cellulose column was achieved by RP-HPLC. The α and the β-globin chains of human and swine Hb can be resolved readily by RP-HPLC. Accordingly, the identification and the stoichiometry of subunits of tetramers present in chromatographic samples was established by RP-HPLC. Vydac C4 column (4.6 × 250 mm) was used for subunit analysis. Chromatograms were developed using a linear gradient of 35–50% acetonitrile containing 0.1% TFA over a period of 100 min. The flow rate was maintained at 1 mL/min.

Oxygen affinity measurements

Oxygen affinity of the parent and hybrid hemoglobins were determined using a Hem-O-Scan. Measurements were made at 37 °C in 50 mM Bis-Tris acetate buffer of required pH and hemoglobin concentration was maintained at 0.5 mM on a tetramer basis.

Subunit exchange assay

The thermodynamic stabilities of the swine–human interspecies hybrids were probed by subunit exchange assay. The human α- and β-chains in the interspecies hybrids were exchanged with their native counterparts. Subunit exchange of interspecies hybrids was monitored by IEF. Hybrids (tetramers) and/or monomers (isolated HMB α or β chains, oxy form, 1:1 or 1:4 molar ratio) were mixed together at a concentration of 8–10 mg/mL in 0.2 M sodium acetate buffer of desired pH. The buffers contained 1 mM EDTA, 1 mM DTT, 1 μg/mL catalase. The mixture was incubated at room temperature for 24 h. Exchange reactions were also performed in 0.9 M $MgCl_2$, pH 7.4 (conditions to form $\alpha\beta$ dimer). Subsequently, the reaction mixtures were analyzed on agarose IEF gels with a blend of pH 6–8 resolve ampholytes. Gels were subjected to IEF at 10 °C for 60 min to resolve the tetramers and monomers.

Stability of hybrid tetramers and dimers

Stability of the heterodimers was checked by gel filtering the hybrids on Superose-12 (FPLC) column using 0.9 M $MgCl_2$, pH 7.4, as the eluent. Under these conditions, Hb tetramer, dimer, and monomers are well resolved on this column. We also compared the V8 proteolytic digestion patterns of human α-chain, HbA, swine Hb, and $^{P}\alpha_2{}^{H}\beta_2$ hybrid at 37 °C, pH 7.8, in 50 mM NH_4HCO_3.

First derivative UV spectra of HbA, swine Hb, and chimeric Hbs

The first derivative spectra of proteins in the UV region (260–340-nm region) in the oxy and deoxy conformation were recorded using a Shimdzu UV 265 spectrometer (Rao et al., 1995). The protein concentration was maintained at 55 μM on heme basis, and the solution conditions for these studies were 50 mM Bis-Tris, pH 7.4, at 25 °C. Spectra of proteins were recorded in the first derivative mode. Protein solutions were deoxygenated by repeated evacuation and flushing with pure nitrogen.

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