Correct assembly of human normal adult hemoglobin when expressed in transgenic swine: chemical, conformational and functional equivalence with the human-derived protein

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Structural and functional investigations of recombinant human hemoglobin A (HbA) isolated from the erythrocytes of transgenic swine coexpressing human α- and β-globins have been carried out to authenticate its correct expression, post-translational processing and assembly. The HbA expressed in transgenic swine (TgHbA) is indistinguishable from the human-derived HbA in terms of its isoelectric pH, mass and elution pattern on a Mono S column. The chemical identity of the α- and β-globin chains of TgHbA with the corresponding chains from human-derived HbA has been established by tryptic peptide mapping and amino acid sequencing. The proton NMR spectra of TgHbA have been established by tryptic peptide mapping and amino acid sequencing. The proton NMR spectra of TgHbA have shown that the conformational aspects of the protein around the heme pocket are indistinguishable from those of the control sample of HbA. The equivalence of the hydrogen bond pattern of TgHbA (in particular the inter-subunit surfaces) with that of authentic HbA has also been established by NMR studies. Consistent with these structural and conformational analyses, the TgHbA also exhibits complete functional equivalence with the human-derived HbA with respect to oxygen affinity, cooperativity, Bohr effect and allosteric properties. Hence the studies presented here demonstrate that the transgenic swine system correctly transcribes the α- and β-globin transgenes, translates the respective α- and β-globin mRNA to generate the corresponding globin chains, carries out the correct cotranslational processing of the translated globin chains, inserts the heme into the globin chains in the same orientation as in the human-derived HbA and assembles the α- and β-subunits into a functionally cooperative tetramer that exhibits a response to allosteric effectors identical with that of human-derived HbA. Thus, in the transgenic swine system, in vitro chemical manipulation steps such as those needed in the Escherichia coli and the yeast systems, to convert the rHbA expressed in these systems into forms functionally identical with that of the human-derived protein, are not needed. An additional advantage of the transgenic swine system is the stability of the transgenes over many generations. Hence the transgenic swine could serve as an excellent system for the production of human HbA (or its variants) for structure–function studies and for therapeutic applications.

Keywords: human normal adult hemoglobin assembly/transgenic swine

Introduction

With the advances in recombinant DNA technology, several systems have been described for the expression of hemoglobin (Nagai and Thogersen, 1984; Nagai et al., 1986; Hoffman et al., 1990; Wagenbach et al., 1991; Doyle et al., 1992; Looker et al., 1992; Shen et al., 1993, 1997; Ogden et al., 1994; Hernan and Sligar, 1995; Dieryck et al., 1997). Expression of human hemoglobin in heterologous systems not only opens up a new frontier for structure–function relationship studies of Hb in unprecedented detail, but also provides an elegant approach to produce human pathogen-free hemoglobin for applications in therapeutic use. It is conceivable that recombinant hemoglobins could be engineered to exhibit the desired properties: designer hemoglobins. Expression of human Hb chains in a heterologous system was first reported by Nagai and Thogersen (1984, 1987). Their system involved expression of the β-globin chain as a fusion protein in Escherichia coli. The fusion protein was purified and cleaved with factor Xa to generate the correct amino terminus for the β-chain. This β-globin was then mixed with the normal human α-chain and assembled by the allopex intermediate pathway into a cooperative semi-recombinant HbA. The characterization of this protein by X-ray crystallography, Raman spectroscopy and CO recombination kinetics established that the behavior of the semi-recombinant Hb is identical with that of human-derived Hb (Nagai et al., 1986).

Subsequently, expression systems for the preparation of a fully recombinant HbA have been developed by coexpressing the α- and β-chains in E.coli (Hoffman et al., 1990; Hernan et al., 1992, Looker et al., 1992). However, the expressed recombinant α- and β-globins contained an additional methionine residue at their respective amino termini. To circumvent this inability of E.coli to process the initiator methionine of both recombinant Hb chains, several investigators (Doyle et al., 1992; Hernan et al., 1992, Kavanaugh et al., 1992; Looker et al., 1992; Shen et al., 1993, Hernan and Sligar, 1995) have constructed N-terminal mutations either in the β-chain alone or in both the α- and the β-chains. The fully recombinant systems described by Looker et al. (1992) and Hernan and Sligar (1995) produce a truncated rHbA, i.e. des-Val Hb, in which valine, the normal N-terminal amino acid residue in both the α- and β-chains, has been deleted and replaced by methionines. The oxygen equilibrium binding properties of this rHb showed a reduced Hill coefficient, Bohr effect and inositol hexaphosphate (IHP) effect as compared with human HbA (Looker et al., 1992; Hernan and Sligar, 1995). This is not surprising because the native N-terminal valine residues of the α- and β-chains of HbA are known to play important roles in regulating oxygen affinity, interactions with allosteric effectors and the Bohr effect (Bunn and Forget, 1986). It should be noted that the expressed Hb prepared by Hernan and Sligar (1995) is the same as rHb0.0 reported by Looker et al. (1992).

An elegant alternative strategy has been developed by Shen...
et al. (1993) to maintain the integrity of the N-terminal amino acids of the expressed Hbs. Their strategy involved over-expression of methionine aminopeptidase in E.coli. In this system, processing of the amino terminus of the globin chains proceeded correctly, as in the human system, and hence authentic HbA was produced.

In contrast to E.coli, yeast appears to have sufficient enzymes to process the initiator methionine appropriately just as the mammalian systems. In view of this advantage, globin genes have been expressed in the yeast system in order to achieve the correct amino terminal cotranslational processing of the expressed chains (Wagenbach et al., 1991; Adachi et al., 1992; Ogden et al., 1994). However, the yield of the expressed Hb in yeast is low.

Recent advances in our understanding of the globin gene regulation, particularly the discovery of the upstream enhancer in yeast is low. Ogden et al. expressed chains (Wagenbach et al., 1994). However, the yield of the expressed Hb in yeast is low.

Materials and methods

Purification of Hb

Human HbA was purified from the erythrocyte lysates by DEAE-cellulose chromatography followed by a second chromatography on CM-cellulose, as described earlier (Acharya et al., 1983). Production of transgenic swine expressing human HbA and procedures for the large-scale purification of the recombinant HbA (TgHbA) have been described previously (Swanson et al., 1992; Garg et al., 1993; Kumar 1995). For the characterizations described here, the TgHbA was purified from lysates of erythrocytes from transgenic swines expressing HbA by a combination of the same DEAE- and CM-cellulose chromatographic procedures as described above for the human HbA. The protein thus obtained was >99% pure, as estimated from isoelectric focusing on an Isolab Resolve gel system.

1H-NMR spectroscopy

1H-NMR experiments were performed on a Bruker AM-300 spectrometer operating at 300.15 MHz and 29°C, using a 5 mm multinuclear probe. The Hb samples were of 4–7% concentration in 0.1 M sodium phosphate, pH 7.0. To suppress the water signal effectively, a jump-and-return pulse sequence was used (Plateau and Gueron, 1982). The HbCO and HbO2 spectra have spectral widths of 8 kHz and 8000 data points; 1024 scans were averaged for each spectrum. The deoxy-Hb spectrum has a spectral width of 71.4 kHz for inclusion of the hyperfine-shifted NH proximal histidyl resonances. Typically, 8000 or 32 000 scans were averaged for each spectrum. All proton chemical shifts are referenced to the methyl proton resonance of sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS). The references were first obtained from the water signal, which occurs at 4.76 ppm downfield from DSS at 29°C and converted to reference DSS on the plots.

Oxygen affinity measurements

Oxygen dissociation curves for TgHbA were measured using either a Hem-O-Scan (Aminco) or a Hemox-Analyzer (TCS Medical Products, Huntington Valley, PA). The partial oxygen pressure at 50% saturation ($P_{50}$) and the Hill coefficient ($n_{max}$) were determined from each oxygen dissociation curve.

Other methods

Mass spectra of the subunits of recombinant HbA were obtained by electrospray mass spectrometric analysis (carried out at M-Scan, Westchester, PA).

Results

Purification and chemical characterization of TgHbA

Lysate from erythrocytes of the transgenic swine expressing HbA was subjected to DEAE-cellulose chromatography to separate the recombinant HbA from the swine hemoglobin and the inter-species hybrid hemoglobins (Figure 1). The expression level of HbA in the transgenic swine was of the order of 10% of the total hemoglobin in the erythrocytes. On the DEAE-cellulose column, HbA elutes ahead of swine Hb and the inter-species hybrid, human α- and swine β-chains [the other hybrid involving the swine α-chain and human β-chain is not generated in vivo in the transgenic swine (Swanson et al., 1992)]. The peak corresponding to HbA was further purified by rechromatography on CM-cellulose (Figure 1, inset). The TgHbA thus purified was found to be >99% homogeneous by IEF (Figure 2, inset). The TgHbA also eluted as a single homogeneous peak by chromatography on a Mono S column (Pharmacia), at a position corresponding to that of authentic HbA (Figure 2). Chemical characterization studies have revealed that the TgHbA has a normal tetrameric structure.
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as well as the correct stoichiometry of the α- and the β-globin chains as the human-derived HbA (Rao et al., 1994). These studies have also provided evidence for correct cotranslational processing of the amino-terminal regions and absence of post-translational modification of the human globin chains expressed in the transgenic swine and strongly support the contention that the primary structures of the α- and β-globin chains of TgHbA are identical with the corresponding chains of human HbA.

Conformational analysis of TgHbA by 1H-NMR studies

Proton NMR spectra of TgHbA and human HbA in the oxy, CO and deoxy forms are shown in Figures 3 and 4. The 1H-NMR spectra of TgHbA are identical with those of human-derived HbA. The exchangeable proton resonances in the oxy, CO and deoxy spectra arise from hydrogen bonds in the α-β and α-β interfaces of HbA (Fung and Ho, 1975; Asakura et al., 1976; Russu et al., 1987; Ho, 1992). The ring-current shifted proton resonances in HbO2 and HbCO arise from the protons on the porphyrin ring and from the protons on the amino acid residues situated above and below the heme planes. Some of the ring-current shifted resonances have been assigned to the methyl protons of El1Val in either the α- or β-chains (Lindstrom et al., 1972a,b; Craescu and Mispelter, 1985; Dalvit and Ho, 1985; Ho, 1992). There is no observable difference in the ring-current shifted proton resonances between TgHbA and human HbA. Figure 4A shows the very low-field proton resonances (60–80 ppm from DSS) in the deoxy form of TgHbA and human HbA. The resonance at ~63 ppm has been assigned to the hyperfine-shifted NδH-exchangeable proton of the proximal histidine residue of the α-chain of deoxy-HbA and that at ~77 ppm has been assigned to the corresponding residue of the β-chain of deoxy-HbA (La Mar et al., 1980; Takahashi et al., 1980). There is no observable difference in the chemical shift in these two resonances between TgHbA and human HbA. This suggests that the conformation in the proximal histidyl residues is the same between TgHbA and human HbA. Figure 4B gives both the hyperfine-shifted and exchangeable proton resonances of TgHbA and human HbA in the deoxy form over the spectral region 10–25 ppm from DSS.
Fig. 5. Oxygen affinity of TgHbA and its modulation by pH and allosteric effectors. The oxygen affinity measurements were carried out in a Hem-O-Scan in 50 mM Bis-Tris buffer at pH 7.4 and 37°C, at protein concentrations of 0.5 mM. (A) Oxygenation curves: (——) TgHbA; (-----) human HbA. (B) Hill plot: (○) TgHbA; (●) human HbA. (C) Influence of pH on the oxygen affinity (Bohr effect) of (□) TgHbA and (●) human HbA. (D) Influence of 2,3-DPG: (○) TgHbA; (●) human HbA. (E) Influence of chloride on the oxygen affinity of (□) TgHbA and (●) control HbA. In A, D and E, the buffer pH was 7.4 and measurements in A, C and D were in the absence of chloride ions.

DSS. This spectral region gives both hyperfine-shifted proton resonances (11–14 ppm) and the exchangeable proton resonances (11–14 ppm) (Ho, 1992). Again, there is no observable difference in the ferrous hyperfine shifted resonances between TgHbA and human HbA, suggesting no alterations in the heme pockets between these Hbs in the deoxy state (Takahashi et al., 1980; Ho, 1992). The exchangeable proton resonances over the spectral region 11–14 ppm are excellent markers for the quaternary structure (α1β1 and α1β2 subunit interfaces) of an Hb molecule (Fung and Ho, 1975; Ho, 1992). The resonance at ~14 ppm has been assigned to the inter-subunit H-bond between α42 Tyr and β99 Asp [a characteristic feature of the deoxy quaternary structure (Fung and Ho, 1975)]. There is no observable difference at ~14 ppm and other exchangeable resonances shown, suggesting that there is no alteration in the α1β1 and α1β2 subunit interfaces. Hence the data presented here demonstrate that there are no discernible differences in the conformational features of the HbA expressed in transgenic swine relative to human HbA, either in the heme pocket or at the subunit interfaces, in both T- and R-states.

Functional equivalence of TgHbA with human HbA

The oxygen affinity of TgHbA and its modulation by allosteric effectors were compared with those of human-derived HbA to establish the functional equivalence of the two proteins. The measurements were made in a Hem-O-Scan in 50 mM Bis-Tris buffer, pH 7.4 (no chloride) at a tetramer concentration of 0.5 mM and at 37°C. The oxygen affinity of TgHbA, the cooperativity of oxygen binding and the influence of pH on the oxygen affinity (Bohr effect) are essentially indistinguishable from those of the human-derived HbA (Figure 5A, B and C, respectively). Similarly, the modulation of the oxygen affinity of TgHbA by the allosteric effectors 2,3-DPG (Figure 5D) and chloride (Figure 5E) are also indistinguishable from the human-derived HbA. These results confirm that the human α- and β-globins expressed in the swine assemble properly to generate a protein that is functionally equivalent to that assembled in the human system in terms of both its oxygen affinity and its modulation by the allosteric effectors.

The oxygen dissociation curves of TgHbA were also investigated in 100 mM phosphate buffer using a Hemox-Analyzer. In this system, the oxygen affinity of a more dilute solution of Hb (0.1 mM per heme or 0.025 mM per tetramer) is used as compared with the higher protein concentration (0.5 mM per tetramer) used in the Hem-O-Scan. As can be seen in Figure 6, the binding curves of TgHbA and human HbA are superimposable, just as in the Bis-Tris buffer system. Table I lists the Hill coefficients and partial oxygen pressure at 50% saturation ($P_{50}$) of HbA from transgenic swine and human, measured at three different pHs. The values are identical within the allowance of experimental errors. Hence all these structural, conformational and functional studies of TgHbA together establish the equivalence of the transgenic swine-derived HbA with the human-derived HbA.

Stability of the human globin genes in swine

The process of pronuclear micro-injection employed to produce transgenic livestock does not permit targeted insertion of the transgenes; the introduced transgenes are randomly inserted into the animal genome, often as a tandem array. As a result, a stable transmission of these genes in a predictable manner cannot always be assured (Grosveld and Kollias, 1992). Hence, with a view to establishing the stability of the transgenes introduced into swine, one line of transgenic pigs was bred...
through three generations by mating the first- or second-generation transgenic pigs with non-transgenic pigs (Sharma et al., 1993). In the resulting progeny, the levels of expression of HbA and the inter-species hybrid (human α swine β) were measured as a function of lineage and age of the transgenic animal. The results revealed that the second- and third-generation transgenic animals expressed HbA and the hybrid HB at the same level as the parent (‘founder’) transgenic swine and the relative proportion of swine, human and the hybrid Hbs remained unchanged. Similarly, the relative expression level remained consistent at different ages (animals from 2 days to 18 months old were examined).

In another line of transgenic swine (line 38-4), we generated transgenic homozygous animals by breeding second-generation male and female descendants of the founder. The homozygous progeny produced nearly twice as much human hemoglobin as their heterozygous parent, consistent with the doubling of the number of gene copies in the homozygous animal (Sharma et al., 1993). These results suggest that in these lines of transgenic pigs, the α- and β-globin genes are faithfully transmitted and stably expressed. Additionally, in line 38-4, the globin genes are probably integrated in a non-essential or redundant genomic location, such that its disruption in the homozygous animals was not detrimental to the health or survival of the animals.

The physico-chemical properties of transgenic hemoglobin in three generations of line 38-4 animals were investigated to authenticate the stability of the protein. The isoelectric focusing behavior, the mass of the protein and the mass of the purified α- and β-globins (Table II), were identical with those of human-derived hemoglobin and the respective globin chains. These data suggest that the transgenes do not suffer a higher rate of mutation and the protein coding sequences are retained intact in the transgenic progeny. Further support for these data was obtained by comparative tryptic peptide mapping of the globin chains (data not shown). These data, in conjunction with the studies of DNA hybridization and cDNA cloning (data not shown), confirm that human genes and their expression are stably inherited in transgenic pigs.

Discussion

The results of the present study on the recombinant HbA generated in transgenic swine authenticate that the transgenes of globin are expressed and processed correctly in the swine system. The FPLC analysis on a Mono S column has confirmed the chromatographic identity of the TgHbA purified on a DE-52 column with a similarly purified sample of human HbA. The Mono S column has been shown to distinguish between the correctly folded and misfolded forms of HbA (Adachi et al., 1992; Shen et al., 1993, 1997). Adachi et al. (1992) have reported that the rHbA obtained from their yeast expres-

<table>
<thead>
<tr>
<th>Source of Hb</th>
<th>Generation</th>
<th>Molecular mass of globin chains</th>
</tr>
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<tbody>
<tr>
<td>Human</td>
<td></td>
<td>α-Globin: 15126.43, β-Globin: 15867.27</td>
</tr>
<tr>
<td>Pig 38.4</td>
<td>First (G0)</td>
<td>α-Globin: 15126.32, β-Globin: 15867.19</td>
</tr>
<tr>
<td>Pig 149-2</td>
<td>Second (G1)</td>
<td>α-Globin: 15126.45, β-Globin: 15866.92</td>
</tr>
<tr>
<td>Pig 149-2-1</td>
<td>Third (G2)</td>
<td>α-Globin: 15126.36, β-Globin: 15867.02</td>
</tr>
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</table>

Correct assembly of transgenic swine-derived HbA

Although it is generally accepted that all the structural information needed to generate a biologically active protein is encoded in the primary structure of the polypeptide chain (Kim and Baldwin, 1990), it is also becoming increasingly clear that the pathway of acquiring the tertiary and quaternary structures by the polypeptide chains is a flexible one and is very sensitive to the environmental conditions. The role of
other protein factors, namely chaperons, in facilitating the proper assembly of protein, particularly the multimeric proteins, has been recognized (Carillo et al., 1992; Hendrick and Hartl, 1995). Accordingly, just as with the genetic engineering of E.coli for over-expression of methionine aminopeptidase (Shen et al., 1993), further engineering of this system with genes for appropriate chaperons may assure correct assembly of the α- and β-globin chains or their mutant forms in this heterologous system (Cole, 1996).

The studies described here on the recombinant HbA expressed in transgenic swine demonstrate that, unlike the other recombinant systems, the assembly of the α- and β-subunits has proceeded correctly in this system, yielding a tetramer that is conformationally and functionally equivalent to the human-derived HbA. Hence, additional manipulations such as those needed to correct the misassembly in the E.coli and the yeast systems are not necessary in the transgenic swine system. The transgenic swine system therefore appears to be a very reliable expression system to produce authentic HbA and/or rationally designed mutant HB with desired oxygen binding properties for structure–function studies and for potential therapeutic applications (Kumar, 1995). The stability of the integrated transgenes of human HbA for the number of generations of the swine tested so far further enhances the strength of this expression system.

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References
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