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Sligar et al.

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(54) **OXYGEN-BINDING HEME PROTEINS INCORPORATING CIRCULARLY-PERMUTED GLOBINS**

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(73) Assignee: **The Board of Trustees of the University of Illinois**, Urbana, IL (US)

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§ 102(e) Date: **Jun. 28, 1999**

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PCT Pub. Date: **Apr. 2, 1998**

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(51) **Int. Cl.**[7] **C12P 21/06**; C07K 17/00; C07K 14/805

(52) **U.S. Cl.** **435/69.6**; 435/252.3; 435/320.1; 435/325; 536/23.5; 530/385

(58) **Field of Search** 530/385; 536/23.5; 435/69.6, 320.1, 325, 252.3

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(74) *Attorney, Agent, or Firm*—Woodard, Emhardt, Naughton, Moriarty & McNett

(57) **ABSTRACT**

Described are preferred oxygen-binding heme proteins which include at least one hemoglobin molecule incorporating at least one circularly permuted globin, especially an alpha globin. More preferred heme proteins of the invention include high molecular weight hemoglobin multimers. Also described are polynucleotides encoding proteins of the invention, and vectors and host cells including the same.

28 Claims, 21 Drawing Sheets

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Figure 1

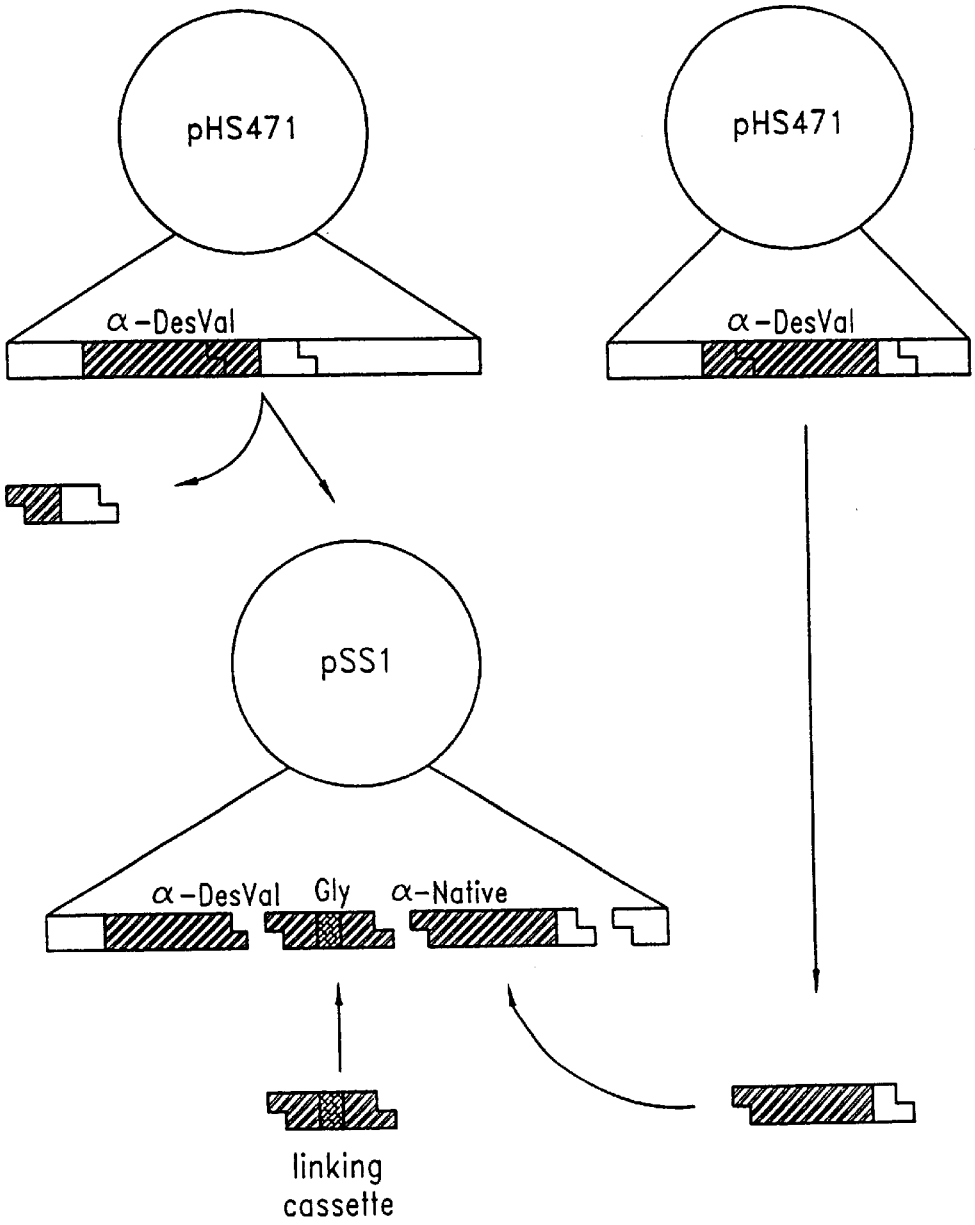
pHS471
α-DesVal
pHS471
α-DesVal
pSS1
α-DesVal
Gly
α-Native
linking
cassette

Figure 2

Di-alpha linking cassette

```
5'-TCGACTGTTCTGACTTCTAAATACCGCGGTGTTCTGTCTCCGGCAGACAAAACTAACGTTAAAGCTGCTTGGGGT-
    3'-GACAAGACTGAAGATTTATGGCGCCACAAGACAGAGGCCGTCTGTTTTGATTGCAATTTCGACGAACCCCA-

  -AAAGTTGGAGCT-3'
  -TTTCAACC-5'
```

Figure 3

Di-alpha Sequence

XbaI 1/37
TCT AGA ATA ACT AAC TAA AGG AGA ACA ACA ACC ATG CTG TCT CCG GCA GAC AAA ACT AAC
AGA TCT TAT TGA TTG ATT TCC TCT TGT TGT TGG TAC GAC AGA GGC CGT CTG TTT TGA TTG
met leu ser pro ala asp lys thr asn

10/64 20/94
GTT AAA GCT GCT TGG GGT AAA GTT GGA GCT CAT GCT GGT GAA TAC GGT GCT GAA GCA CTC
CAA TTT CGA CGA ACC CCA TTT CAA CCT CGA GTA CGA CCA CTT ATG CCA CGA CTT CGT GAG
val lys ala ala trp gly lys val gly ala his ala gly glu tyr gly ala glu ala leu

30/124 40/154
GAG CGT ATG TTC CTG TCT TTC CCG ACT ACT AAA ACG TAC TTC CCG CAT TTC GAC CTG TCT
CTC GCA TAC AAG GAC AGA AAG GGC TGA TGA TTT TGC ATG AAG GGC GTA AAG CTG GAC AGA
glu arg met phe leu ser phe pro thr thr lys thr tyr phe pro his phe asp leu ser

50/184 60/214
CAT GGA TCC GCT CAG GTT AAA GGT CAT GGT AAA AAA GTT GCT GAC GCG TTG ACT AAC GCT
GTA CCT AGG CGA GTC CAA TTT CCA GTA CCA TTT TTT CAA CGA CTG CGC AAC TGA TTG CGA
his gly ser ala gln val lys gly his gly lys lys val ala asp ala leu thr asn ala

70/244 80/274
GTT GCT CAT GTT GAC GAC ATG CCG AAC GCT CTG TCC GCT CTG TCA GAT CTT CAT GCT CAT
CAA CGA GTA CAA CTG CTG TAC GGC TTG CGA GAC AGG CGA GAC AGT CTA GAA GTA CGA GTA
val ala his val asp asp met pro asn ala leu ser ala leu ser asp leu his ala his

90/304 100/334
AAA CTG CGC GTT GAC CCG GTA AAC TTC AAG CTT CTG TCT CAT TGC CTG CTG GTT ACT CTG
TTT GAC GCG CAA CTG GGC CAT TTG AAG TTC GAA GAC AGA GTA ACG GAC GAC CAA TGA GAC
lys leu arg val asp pro val asn phe lys leu leu ser his cys leu leu val thr leu

110/364 120/394
GCT GCT CAT CTG CCG GCA GAA TTC ACT CCG GCT GTT CAT GCT TCT CTG GAT AAA TTC CTG
CGA CGA GTA GAC GGC CGT CTT AAG TGA GGC CGA CAA GTA CGA AGA GAC CTA TTT AAG GAC
ala ala his leu pro ala glu phe thr pro ala val his ala ser leu asp lys phe leu

SalI 140/454
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CGA AGA CAC AGC TGA CAA GAC TGA AGA TTT ATG GCG CCA CAA GAC AGA GGC CGT CTG TTT
ala ser val ser thr val leu thr ser lys tyr arg gly val leu ser pro ala asp lys

150/484 *SacI*
ACT AAC GTT AAA GCT GCT TGG GGT AAA GTT GGA GCT CAT GCT GGT GAA TAC GGT GCT GAA
TGA TTG CAA TTT CGA CGA ACC CCA TTT CAA CCT CGA GTA CGA CCA CTT ATG CCA CGA CTT
thr asn val lys ala ala trp gly lys val gly ala his ala gly glu tyr gly ala glu

170/544 180/574
GCA CTC GAG CGT ATG TTC CTG TCT TTC CCG ACT ACT AAA ACG TAC TTC CCG CAT TTC GAC
CGT GAG CTC GCA TAC AAG GAC AGA AAG GGC TGA TGA TTT TGC ATG AAG GGC GTA AAG CTG
ala leu glu arg met phe leu ser phe pro thr thr lys thr tyr phe pro his phe asp

Figure 3 (cont.)

```
    190/604                                         200/634
CTG TCT CAT GGA TCC GCT CAG GTT AAA GGT CAT GGT AAA AAA GTT GCT GAC GCG TTG ACT
GAC AGA GTA CCT AGG CGA GTC CAA TTT CCA GTA CCA TTT TTT CAA CGA CTG CGC AAC TGA
leu ser his gly ser ala gln val lys gly his gly lys lys val ala asp ala leu thr

    210/664                                         220/694
AAC GCT GTT GCT CAT GTT GAC GAC ATG CCG AAC GCT CTG TCC GCT CTG TCA GAT CTT CAT
TTG CGA CAA CGA GTA CAA CTG CTG TAC GGC TTG CGA GAC AGG CGA GAC AGT CTA GAA GTA
asn ala val ala his val asp asp met pro asn ala leu ser ala leu ser asp leu his

    230/724                                         240/754
GCT CAT AAA CTG CGC GTT GAC CCG GTA AAC TTC AAG CTT CTG TCT CAT TGC CTG CTG GTT
CGA GTA TTT GAC GCG CAA CTG GGC CAT TTG AAG TTC GAA GAC AGA GTA ACG GAC GAC CAA
ala his lys leu arg val asp pro val asn phe lys leu leu ser his cys leu leu val

    250/784                                         260/814
ACT CTG GCT GCT CAT CTG CCG GCA GAA TTC ACT CCG GCT GTT CAT GCT TCT CTG GAT AAA
TGA GAC CGA CGA GTA GAC GGC CGT CTT AAG TGA GGC CGA CAA GTA CGA AGA GAC CTA TTT
thr leu ala ala his leu pro ala glu phe thr pro ala val his ala ser leu asp lys

    270/844                                         280/874             PstI
TTC CTG GCT TCT GTG TCG ACT GTT CTG ACT TCT AAA TAC CGT TAA TGA CTG CAG
AAG GAC CGA AGA CAC AGC TGA CAA GAC TGA AGA TTT ATG GCA ATT ACT GAC GTC
phe leu ala ser val ser thr val leu thr ser lys tyr arg OCH OPA
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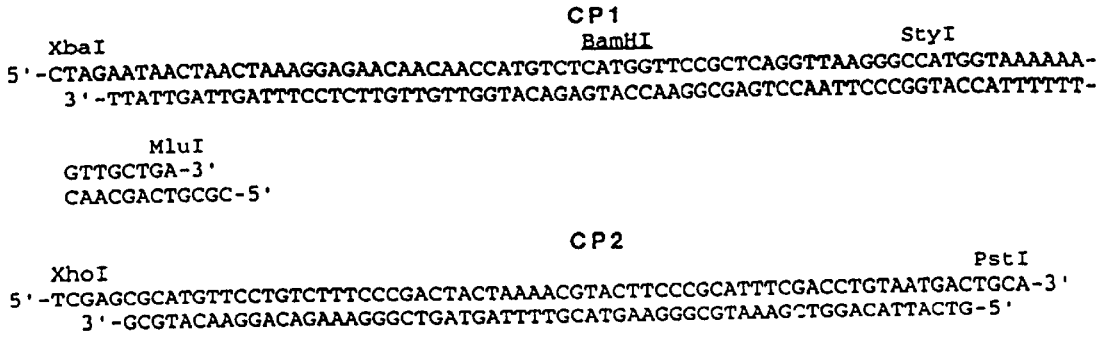
Figure 4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XbaI
BamHI
StyI
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3'-TTATTGATTGATTTCCTCTTGTTGTTGGTACAGAGTACCAAGGCGAGTCCAATTCCCGGTACCATTTTTT-
MluI
GTTGCTGA-3'
CAACGACTGCGC-5'
CP2
PstI
XhoI
5'-TCGAGCGCATGTTCCTGTCTTTCCCGACTACTAAAACGTACTTCCCGCATTTCGACCTGTAATGACTGCA-3'
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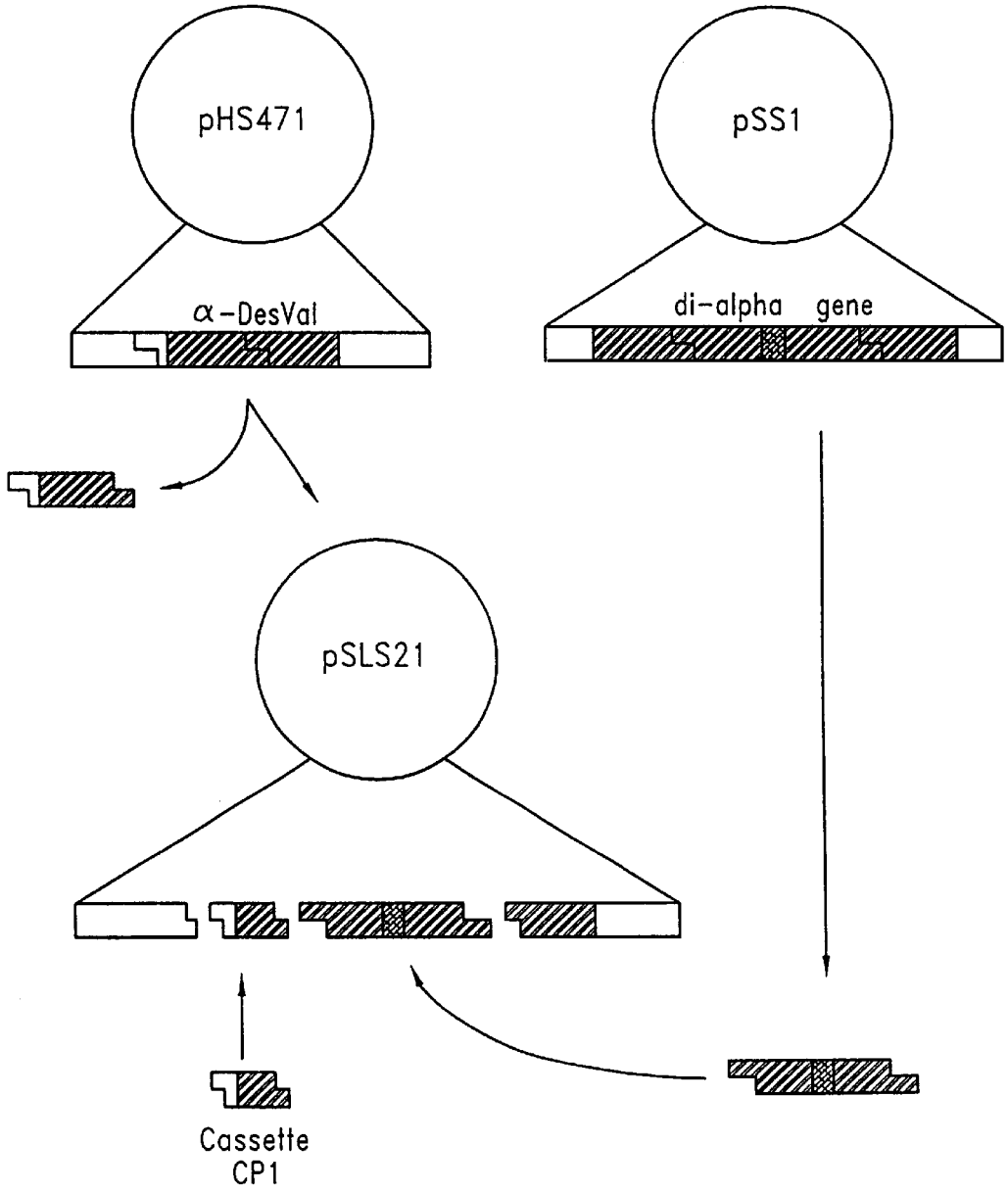
Figure 5



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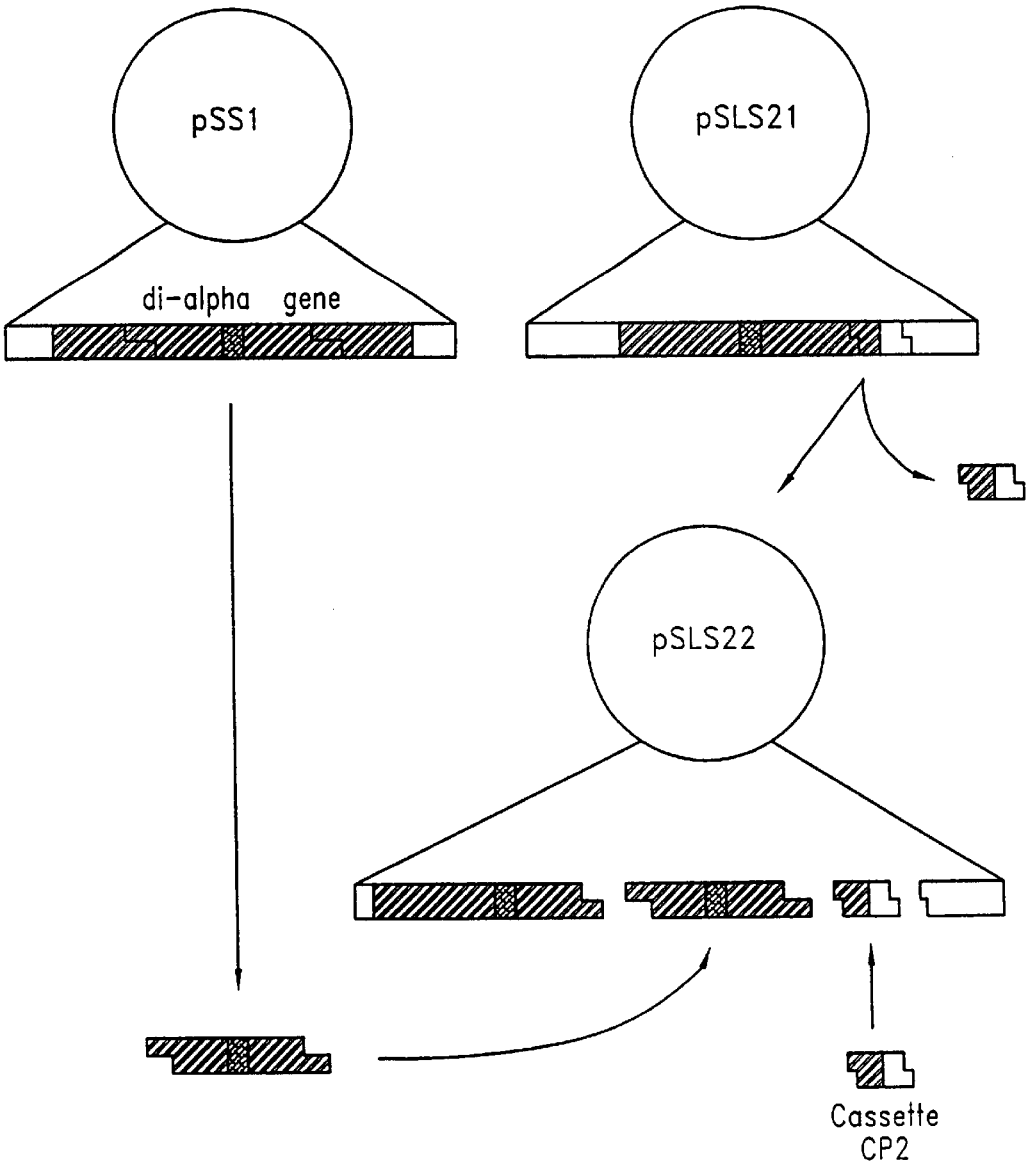

Figure 7

Circularly Permuted Di-alpha Gene Sequence

```
 XbaI                                                    1/37   BamHI
TCT AGA ATA ACT AAC TAA AGG AGA ACA ACA ACC ATG TCT CAT GGT TCC GCT CAG GTT AAG
AGA TCT TAT TGA TTG ATT TCC TCT TGT TGT TGG TAC AGA GTA CCA AGG CGA GTC CAA TTC
                                            met ser his gly ser ala gln val lys

  StyI                          MluI                    20/94
GGC CAT GGT AAA AAA GTT GCT GAC GCG TTG ACT AAC GCT GTT GCT CAT GTT GAC GAC ATG
CCG GTA CCA TTT TTT CAA CGA CTG CGC AAC TGA TTG CGA CAA CGA GTA CAA CTG CTG TAC
gly his gly lys lys val ala asp ala leu thr asn ala val ala his val asp asp met

    30/124                                          40/154
CCG AAC GCT CTG TCC GCT CTG TCA GAT CTT CAT GCT CAT AAA CTG CGC GTT GAC CCG GTA
GGC TTG CGA GAC AGG CGA GAC AGT CTA GAA GTA CGA GTA TTT GAC GCG CAA CTG GGC CAT
pro asn ala leu ser ala leu ser asp leu his ala his lys leu arg val asp pro val

    50/184                                          60/214
AAC TTC AAG CTT CTG TCT CAT TGC CTG CTG GTT ACT CTG GCT GCT CAT CTG CCG GCA GAA
TTG AAG TTC GAA GAC AGA GTA ACG GAC GAC CAA TGA GAC CGA CGA GTA GAC GGC CGT CTT
asn phe lys leu leu ser his cys leu leu val thr leu ala ala his leu pro ala glu

    70/244                                          80/274
TTC ACT CCG GCT GTT CAT GCT TCT CTG GAT AAA TTC CTG GCT TCT GTG TCG ACT GTT CTG
AAG TGA GGC CGA CAA GTA CGA AGA GAC CTA TTT AAG GAC CGA AGA CAC AGC TGA CAA GAC
phe thr pro ala val his ala ser leu asp lys phe leu ala ser val ser thr val leu

    90/304                                          100/334
ACT TCT AAA TAC CGC GGT GTT CTG TCT CCG GCA GAC AAA ACT AAC GTT AAA GCT GCT TGG
TGA AGA TTT ATG GCG CCA CAA GAC AGA GGC CGT CTG TTT TGA TTG CAA TTT CGA CGA ACC
thr ser lys tyr arg gly val leu ser pro ala asp lys thr asn val lys ala ala trp

    110/364                                         120/394
GGT AAA GTT GGA GCT CAT GCT GGT GAA TAC GGT GCT GAA GCA CTC GAG CGT ATG TTC CTG
CCA TTT CAA CCT CGA GTA CGA CCA CTT ATG CCA CGA CTT CGT GAG CTC GCA TAC AAG GAC
gly lys val gly ala his ala gly glu tyr gly ala glu ala leu glu arg met phe leu

    130/424                                         140/454             BamHI
TCT TTC CCG ACT ACT AAA ACG TAC TTC CCG CAT TTC GAC CTG TCT CAT GGA TCC GCT CAG
AGA AAG GGC TGA TGA TTT TGC ATG AAG GGC GTA AAG CTG GAC AGA GTA CCT AGG CGA GTC
ser phe pro thr thr lys thr tyr phe pro his phe asp leu ser his gly ser ala gln

    150/484                                         160/514
GTT AAA GGT CAT GGT AAA AAA GTT GCT GAC GCG TTG ACT AAC GCT GTT GCT CAT GTT GAC
CAA TTT CCA GTA CCA TTT TTT CAA CGA CTG CGC AAC TGA TTG CGA CAA CGA GTA CAA CTG
val lys gly his gly lys lys val ala asp ala leu thr asn ala val ala his val asp

    170/544                                         180/574
GAC ATG CCG AAC GCT CTG TCC GCT CTG TCA GAT CTT CAT GCT CAT AAA CTG CGC GTT GAC
CTG TAC GGC TTG CGA GAC AGG CGA GAC AGT CTA GAA GTA CGA GTA TTT GAC GCG CAA CTG
asp met pro asn ala leu ser ala leu ser asp leu his ala his lys leu arg val asp

    190/604                                         200/634
CCG GTA AAC TTC AAG CTT CTG TCT CAT TGC CTG CTG GTT ACT CTG GCT GCT CAT CTG CCG
GGC CAT TTG AAG TTC GAA GAC AGA GTA ACG GAC GAC CAA TGA GAC CGA CGA GTA GAC GGC
pro val asn phe lys leu leu ser his cys leu leu val thr leu ala ala his leu pro

    210/664                                         220/694
GCA GAA TTC ACT CCG GCT GTT CAT GCT TCT CTG GAT AAA TTC CTG GCT TCT GTG TCG ACT
CGT CTT AAG TGA GGC CGA CAA GTA CGA AGA GAC CTA TTT AAG GAC CGA AGA CAC AGC TGA
ala glu phe thr pro ala val his ala ser leu asp lys phe leu ala ser val ser thr
```

Figure 7 (cont.)

```
    230/724                                     240/754
GTT CTG ACT TCT AAA TAC CGC GGT GTT CTG TCT CCG GCA GAC AAA ACT AAC GTT AAA GCT
CAA GAC TGA AGA TTT ATG GCG CCA CAA GAC AGA GGC CGT CTG TTT TGA TTG CAA TTT CGA
val leu thr ser lys tyr arg gly val leu ser pro ala asp lys thr asn val lys ala

    250/784                                     260/814             XhoI
GCT TGG GGT AAA GTT GGA GCT CAT GCT GGT GAA TAC GGT GCT GAA GCA CTC GAG CGT ATG
CGA ACC CCA TTT CAA CCT CGA GTA CGA CCA CTT ATG CCA CGA CTT CGT GAG CTC GCA TAC
ala trp gly lys val gly ala his ala gly glu tyr gly ala glu ala leu glu arg met

    270/844                                     280/874                     PstI
TTC CTG TCT TTC CCG ACT ACT AAA ACG TAC TTC CCG CAT TTC GAC CTG TAA TGA CTG CAG
AAG GAC AGA AAG GGC TGA TGA TTT TGC ATG AAG GGC GTA AAG CTG GAC ATT ACT GAC GTC
phe leu ser phe pro thr thr lys thr tyr phe pro his phe asp leu OCH OPA
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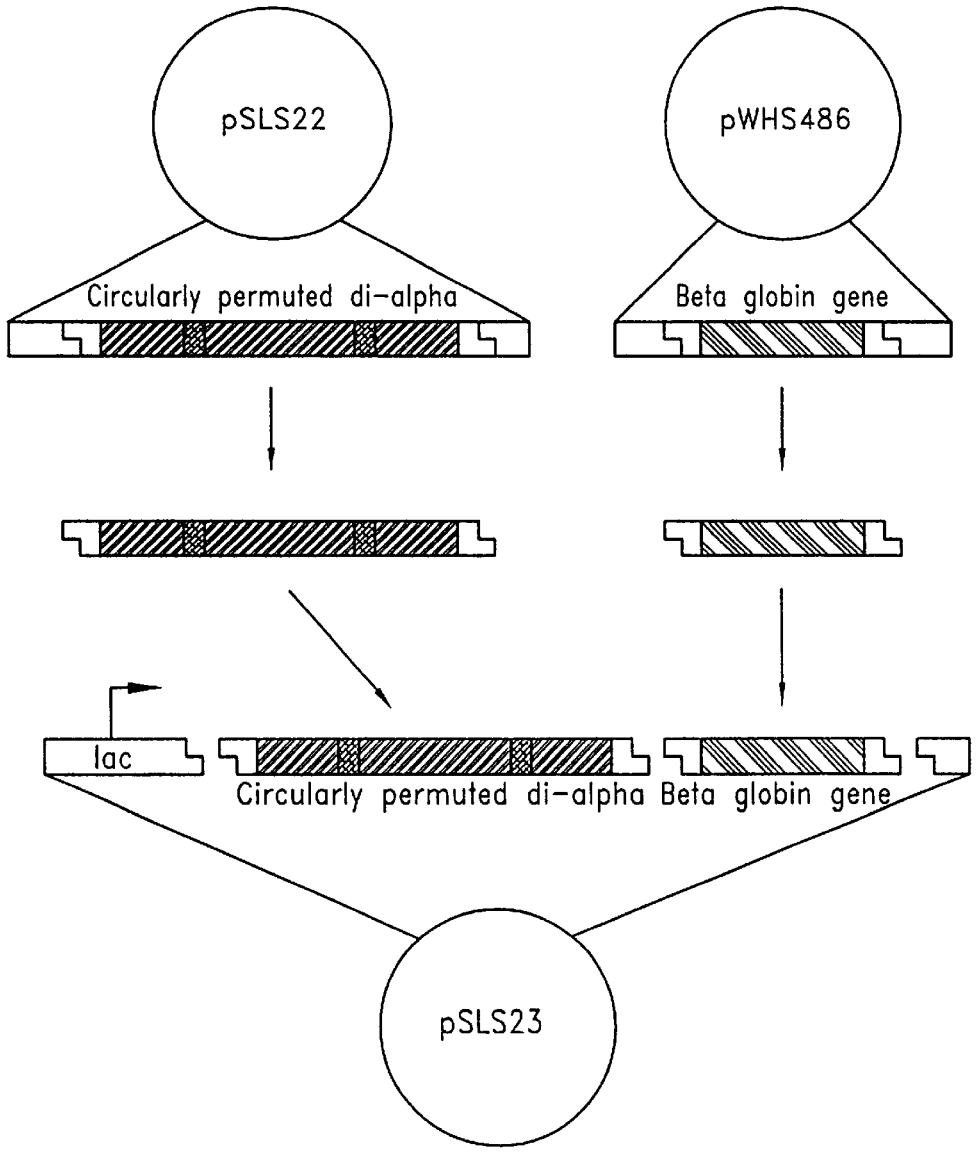
Figure 8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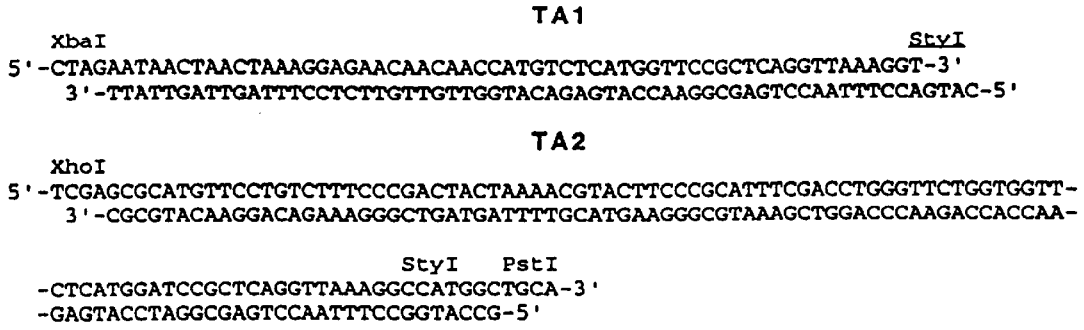
Circularly permuted di-alpha
Beta globin gene
lac
Circularly permuted di-alpha
Beta globin gene
pSLS23

Figure 9


TA1
XbaI
StyI
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3'-TTATTGATTGATTTCCTCTTGTTGTTGGTACAGAGTACCAAGGCGAGTCCAATTTCCAGTAC-5'
TA2
XhoI
5'-TCGAGCGCATGTTCCTGTCTTTCCCGACTACTAAAACGTACTTCCCGCATTTCGACCTGGGTTCTGGTGGTT-
3'-CGCGTACAAGGACAGAAAGGGCTGATGATTTTGCATGAAGGGCGTAAAGCTGGACCCAAGACCACCAA-
StyI PstI
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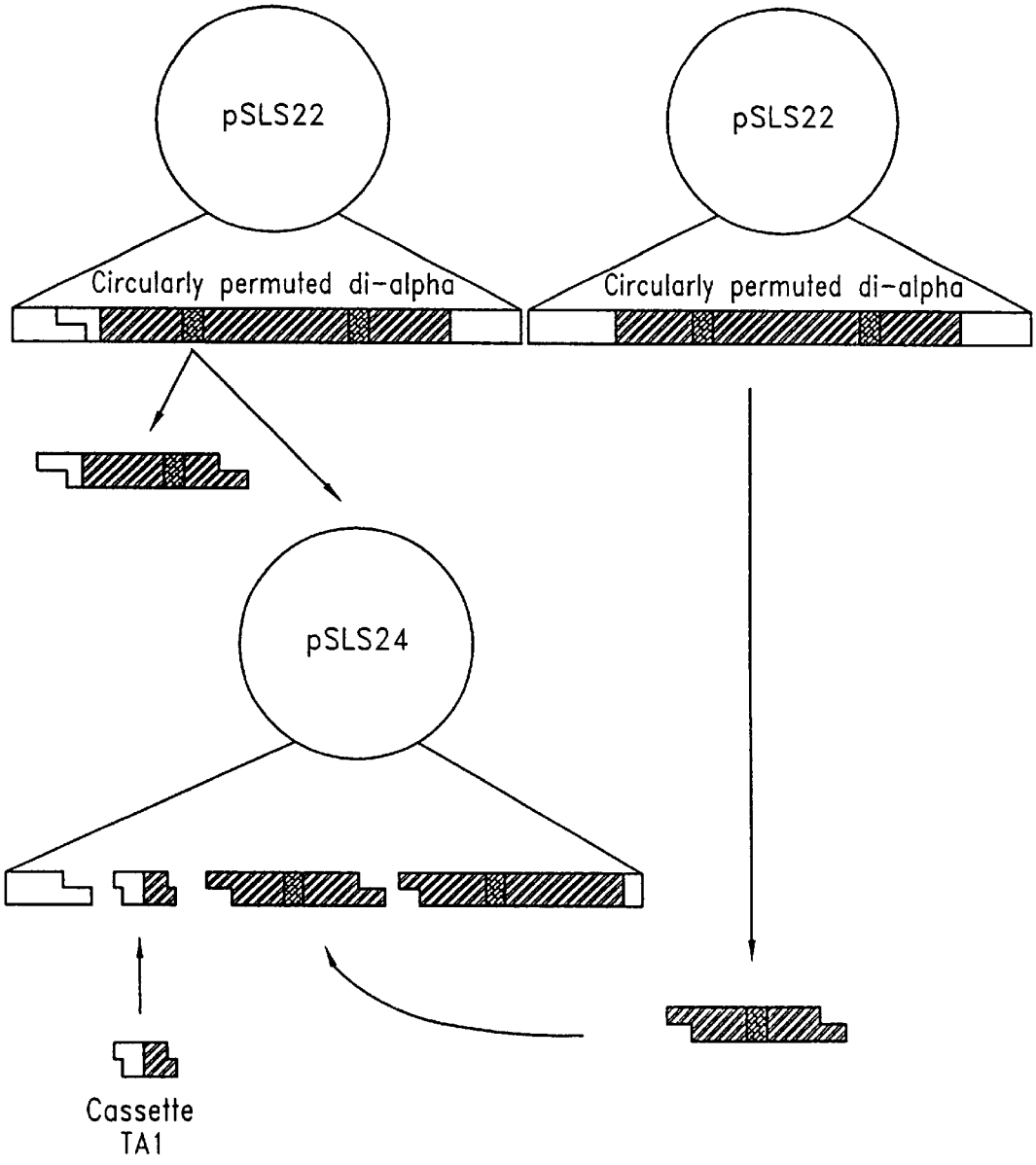
-GAGTACCTAGGCGAGTCCAATTTCCGGTACCG-5'

Figure 10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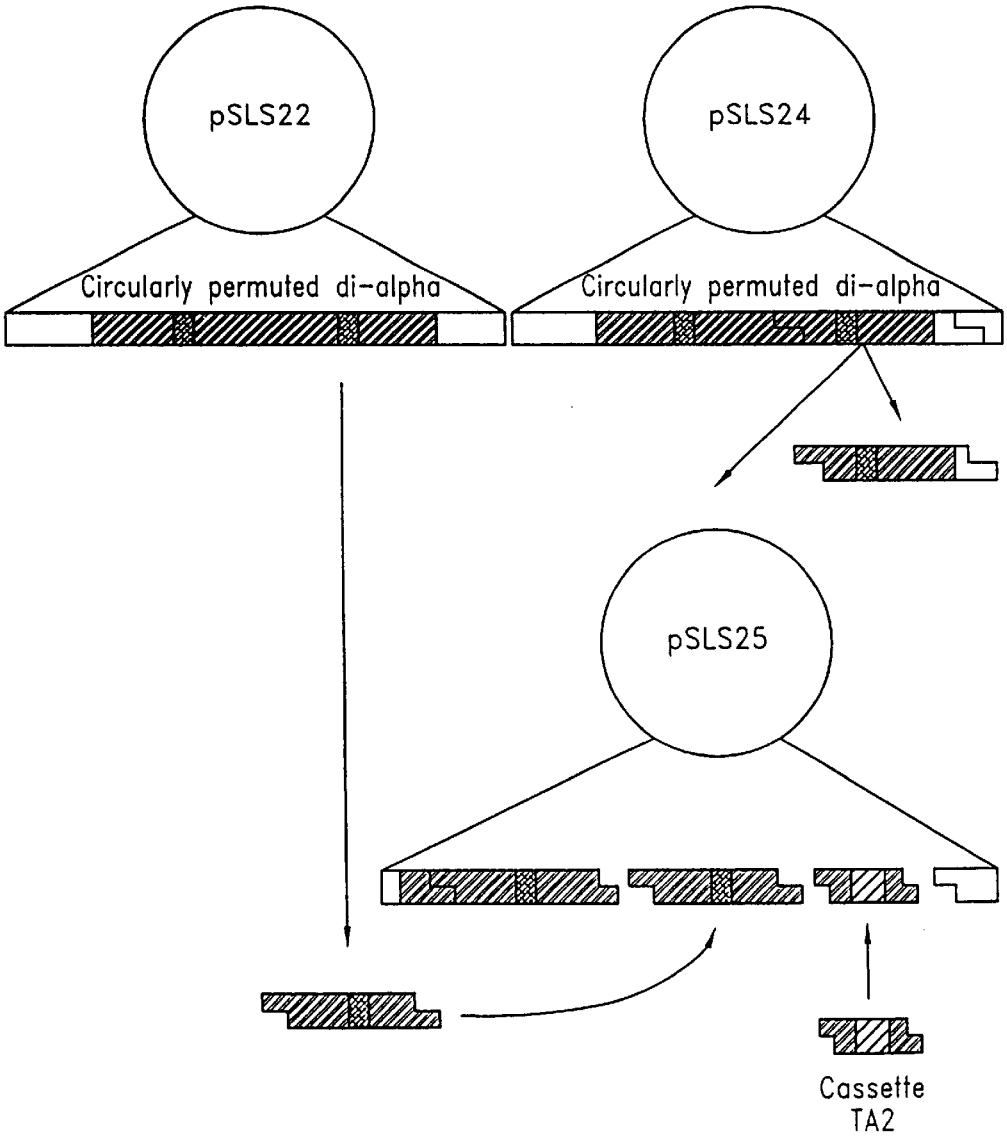
pSLS22
Circularly permuted di-alpha
Circularly permuted di-alpha
pSLS24
Cassette
TA1

Figure 11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Circularly permuted di-alpha
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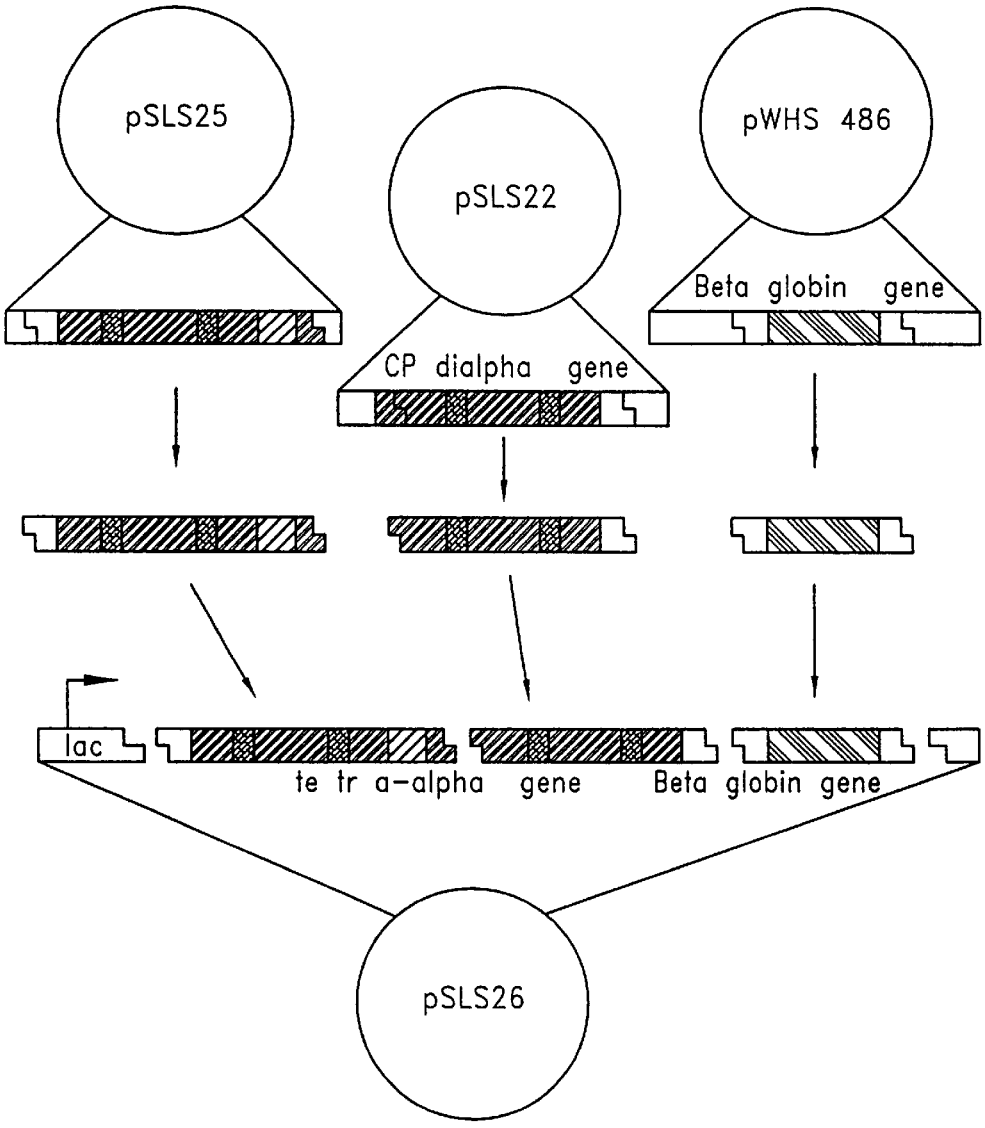
pSLS25
Cassette
TA2

Figure 12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pSLS22
pWHS 486
Beta globin gene
CP dialpha gene
lac
te tr a-alpha gene
Beta globin gene
pSLS26

Figure 13

Circularly Permuted Tetra-alpha Gene Sequence

```
                                                        1/37   BamHI
 XbaI
TCT AGA ATA ACT AAC TAA AGG AGA ACA ACA ACC ATG TCT CAT GGT TCC GCT CAG GTT AAG
AGA TCT TAT TGA TTG ATT TCC TCT TGT TGT TGG TAC AGA GTA CCA AGG CGA GTC CAA TTC
                                            met ser his gly ser ala gln val lys

                                                        20/94
  StyI
GGT CAT GGT AAA AAA GTT GCT GAC GCG TTG ACT AAC GCT GTT GCT CAT GTT GAC GAC ATG
CCG GTA CCA TTT TTT CAA CGA CTG CGC AAC TGA TTG CGA CAA CGA GTA CAA CTG CTG TAC
gly his gly lys lys val ala asp ala leu thr asn ala val ala his val asp asp met

    30/124                                              40/154
CCG AAC GCT CTG TCC GCT CTG TCA GAT CTT CAT GCT CAT AAA CTG CGC GTT GAC CCG GTA
GGC TTG CGA GAC AGG CGA GAC AGT CTA GAA GTA CGA GTA TTT GAC GCG CAA CTG GGC CAT
pro asn ala leu ser ala leu ser asp leu his ala his lys leu arg val asp pro val

    50/184                                              60/214
AAC TTC AAG CTT CTG TCT CAT TGC CTG CTG GTT ACT CTG GCT GCT CAT CTG CCG GCA GAA
TTG AAG TTC GAA GAC AGA GTA ACG GAC GAC CAA TGA GAC CGA CGA GTA GAC GGC CGT CTT
asn phe lys leu leu ser his cys leu leu val thr leu ala ala his leu pro ala glu

    70/244                                              80/274
TTC ACT CCG GCT GTT CAT GCT TCT CTG GAT AAA TTC CTG GCT TCT GTG TCG ACT GTT CTG
AAG TGA GGC CGA CAA GTA CGA AGA GAC CTA TTT AAG GAC CGA AGA CAC AGC TGA CAA GAC
phe thr pro ala val his ala ser leu asp lys phe leu ala ser val ser thr val leu

    90/304                                             100/334
ACT TCT AAA TAC CGC GGT GTT CTG TCT CCG GCA GAC AAA ACT AAC GTT AAA GCT GCT TGG
TGA AGA TTT ATG GCG CCA CAA GAC AGA GGC CGT CTG TTT TGA TTG CAA TTT CGA CGA ACC
thr ser lys tyr arg gly val leu ser pro ala asp lys thr asn val lys ala ala trp

    110/364                                            120/394
GGT AAA GTT GGA GCT CAT GCT GGT GAA TAC GGT GCT GAA GCA CTC GAG CGT ATG TTC CTG
CCA TTT CAA CCT CGA GTA CGA CCA CTT ATG CCA CGA CTT CGT GAG CTC GCA TAC AAG GAC
gly lys val gly ala his ala gly glu tyr gly ala glu ala leu glu arg met phe leu

    130/424                                 140/454                BamHI
TCT TTC CCG ACT ACT AAA ACG TAC TTC CCG CAT TTC GAC CTG TCT CAT GGA TCC GCT CAG
AGA AAG GGC TGA TGA TTT TGC ATG AAG GGC GTA AAG CTG GAC AGA GTA CCT AGG CGA GTC
ser phe pro thr thr lys thr tyr phe pro his phe asp leu ser his gly ser ala gln

    150/484                                            160/514
GTT AAA GGT CAT GGT AAA AAA GTT GCT GAC GCG TTG ACT AAC GCT GTT GCT CAT GTT GAC
CAA TTT CCA GTA CCA TTT TTT CAA CGA CTG CGC AAC TGA TTG CGA CAA CGA GTA CAA CTG
val lys gly his gly lys lys val ala asp ala leu thr asn ala val ala his val asp

    170/544                                            180/574
GAC ATG CCG AAC GCT CTG TCC GCT CTG TCA GAT CTT CAT GCT CAT AAA CTG CGC GTT GAC
CTG TAC GGC TTG CGA GAC AGG CGA GAC AGT CTA GAA GTA CGA GTA TTT GAC GCG CAA CTG
asp met pro asn ala leu ser ala leu ser asp leu his ala his lys leu arg val asp

    190/604                                            200/634
CCG GTA AAC TTC AAG CTT CTG TCT CAT TGC CTG CTG GTT ACT CTG GCT GCT CAT CTG CCG
GGC CAT TTG AAG TTC GAA GAC AGA GTA ACG GAC GAC CAA TGA GAC CGA CGA GTA GAC GGC
pro val asn phe lys leu leu ser his cys leu leu val thr leu ala ala his leu pro

    210/664                                            220/694
GCA GAA TTC ACT CCG GCT GTT CAT GCT TCT CTG GAT AAA TTC CTG GCT TCT GTG TCG ACT
CGT CTT AAG TGA GGC CGA CAA GTA CGA AGA GAC CTA TTT AAG GAC CGA AGA CAC AGC TGA
ala glu phe thr pro ala val his ala ser leu asp lys phe leu ala ser val ser thr
```

Figure 13 (cont.)

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    230/724                                     240/754
GTT CTG ACT TCT AAA TAC CGC GGT GTT CTG TCT CCG GCA GAC AAA ACT AAC GTT AAA GCT
CAA GAC TGA AGA TTT ATG GCG CCA CAA GAC AGA GGC CGT CTG TTT TGA TTG CAA TTT CGA
val leu thr ser lys tyr arg gly val leu ser pro ala asp lys thr asn val lys ala

    250/784                                     260/814             XhoI
GCT TGG GGT AAA GTT GGA GCT CAT GCT GGT GAA TAC GGT GCT GAA GCA CTC GAG CGT ATG
CGA ACC CCA TTT CAA CCT CGA GTA CGA CCA CTT ATG CCA CGA CTT CGT GAG CTC GCA TAC
ala trp gly lys val gly ala his ala gly glu tyr gly ala glu ala leu glu arg met

    270/844                                     280/874
TTC CTG TCT TTC CCG ACT ACT AAA ACG TAC TTC CCG CAT TTC GAC CTG GGT TCT GGT GGT
AAG GAC AGA AAG GGC TGA TGA TTT TGC ATG AAG GGC GTA AAG CTG GAC CCA AGA CCA CCA
phe leu ser phe pro thr thr lys thr tyr phe pro his phe asp leu gly ser gly gly

    280/904                     StyI            290/934
TCT CAT GGT TCC GCT CAG GTT AAG GGC CAT GGT AAA AAA GTT GCT GAC GCG TTG ACT AAC
AGA GTA CCA AGG CGA GTC CAA TTC CCG GTA CCA TTT TTT CAA CGA CTG CGC AAC TGA TTG
ser his gly ser ala gln val lys gly his gly lys lys val ala asp ala leu thr asn

    300/964                                     310/994
GCT GTT GCT CAT GTT GAC GAC ATG CCG AAC GCT CTG TCC GCT CTG TCA GAT CTT CAT GCT
CGA CAA CGA GTA CAA CTG CTG TAC GGC TTG CGA GAC AGG CGA GAC AGT CTA GAA GTA CGA
ala val ala his val asp asp met pro asn ala leu ser ala leu ser asp leu his ala

    320/1024                                    330/1054
CAT AAA CTG CGC GTT GAC CCG GTA AAC TTC AAG CTT CTG TCT CAT TGC CTG CTG GTT ACT
GTA TTT GAC GCG CAA CTG GGC CAT TTG AAG TTC GAA GAC AGA GTA ACG GAC GAC CAA TGA
his lys leu arg val asp pro val asn phe lys leu leu ser his cys leu leu val thr

    340/1084                                    350/1114
CTG GCT GCT CAT CTG CCG GCA GAA TTC ACT CCG GCT GTT CAT GCT TCT CTG GAT AAA TTC
GAC CGA CGA GTA GAC GGC CGT CTT AAG TGA GGC CGA CAA GTA CGA AGA GAC CTA TTT AAG
leu ala ala his leu pro ala glu phe thr pro ala val his ala ser leu asp lys phe

    360/1044                                    370/1174
CTG GCT TCT GTG TCG ACT GTT CTG ACT TCT AAA TAC CGC GGT GTT CTG TCT CCG GCA GAC
GAC CGA AGA CAC AGC TGA CAA GAC TGA AGA TTT ATG GCG CCA CAA GAC AGA GGC CGT CTG
leu ala ser val ser thr val leu thr ser lys tyr arg gly val leu ser pro ala asp

    380/1204                                    390/1234
AAA ACT AAC GTT AAA GCT GCT TGG GGT AAA GTT GGA GCT CAT GCT GGT GAA TAC GGT GCT
TTT TGA TTG CAA TTT CGA CGA ACC CCA TTT CAA CCT CGA GTA CGA CCA CTT ATG CCA CGA
lys thr asn val lys ala ala trp gly lys val gly ala his ala gly glu tyr gly ala

    400/1264                                    410/1294
GAA GCA CTC GAG CGT ATG TTC CTG TCT TTC CCG ACT ACT AAA ACG TAC TTC CCG CAT TTC
CTT CGT GAG CTC GCA TAC AAG GAC AGA AAG GGC TGA TGA TTT TGC ATG AAG GGC GTA AAG
glu ala leu glu arg met phe leu ser phe pro thr thr lys thr tyr phe pro his phe

    420/1324                                    430/1354
GAC CTG TCT CAT GGA TCC GCT CAG GTT AAA GGT CAT GGT AAA AAA GTT GCT GAC GCG TTG
CTG GAC AGA GTA CCT AGG CGA GTC CAA TTT CCA GTA CCA TTT TTT CAA CGA CTG CGC AAC
asp leu ser his gly ser ala gln val lys gly his gly lys lys val ala asp ala leu
```

Figure 13 (cont.)

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    440/1384                                    450/1414
ACT AAC GCT GTT GCT CAT GTT GAC GAC ATG CCG AAC GCT CTG TCC GCT CTG TCA GAT CTT
TGA TTG CGA CAA CGA GTA CAA CTG CTG TAC GGC TTG CGA GAC AGG CGA GAC AGT CTA GAA
thr asn ala val ala his val asp asp met pro asn ala leu ser ala leu ser asp leu

    460/1444                                    470/1474
CAT GCT CAT AAA CTG CGC GTT GAC CCG GTA AAC TTC AAG CTT CTG TCT CAT TGC CTG CTG
GTA CGA GTA TTT GAC GCG CAA CTG GGC CAT TTG AAG TTC GAA GAC AGA GTA ACG GAC GAC
his ala his lys leu arg val asp pro val asn phe lys leu leu ser his cys leu leu

    480/1504                                    490/1534
GTT ACT CTG GCT GCT CAT CTG CCG GCA GAA TTC ACT CCG GCT GTT CAT GCT TCT CTG GAT
CAA TGA GAC CGA CGA GTA GAC GGC CGT CTT AAG TGA GGC CGA CAA GTA CGA AGA GAC CTA
val thr leu ala ala his leu pro ala glu phe thr pro ala val his ala ser leu asp

    500/1564                                    510/1594
AAA TTC CTG GCT TCT GTG TCG ACT GTT CTG ACT TCT AAA TAC CGC GGT GTT CTG TCT CCG
TTT AAG GAC CGA AGA CAC AGC TGA CAA GAC TGA AGA TTT ATG GCG CCA CAA GAC AGA GGC
lys phe leu ala ser val ser thr val leu thr ser lys tyr arg gly val leu ser pro

    520/1624                                    530/1654
GCA GAC AAA ACT AAC GTT AAA GCT GCT TGG GGT AAA GTT GGA GCT CAT GCT GGT GAA TAC
CGT CTG TTT TGA TTG CAA TTT CGA CGA ACC CCA TTT CAA CCT CGA GTA CGA CCA CTT ATG
ala asp lys thr asn val lys ala ala trp gly lys val gly ala his ala gly glu tyr

    540/1584                                    550/1614
GGT GCT GAA GCA CTC GAG CGT ATG TTC CTG TCT TTC CCG ACT ACT AAA ACG TAC TTC CCG
CCA CGA CTT CGT GAG CTC GCA TAC AAG GAC AGA AAG GGC TGA TGA TTT TGC ATG AAG GGC
gly ala glu ala leu glu arg met phe leu ser phe pro thr thr lys thr tyr phe pro

    560/1644          PstI
CAT TTC GAC CTG TAA TGA CTG CAG
GTA AAG CTG GAC ATT ACT GAC GTC
his phe asp leu OCH OPA
```

Figure 14

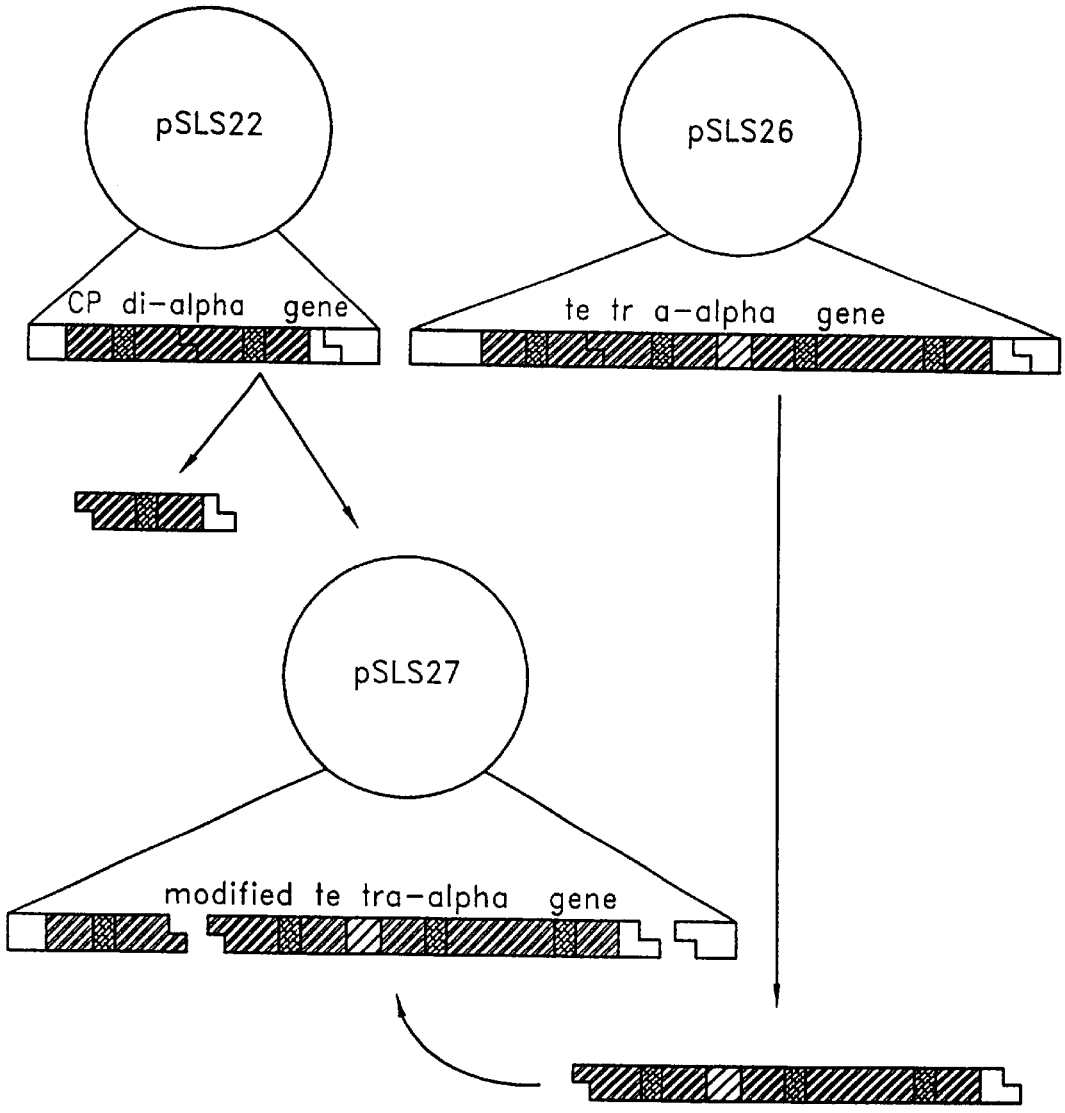

pSLS22
pSLS26
CP di-alpha gene
te tr a-alpha gene
pSLS27
modified te tra-alpha gene

Figure 15

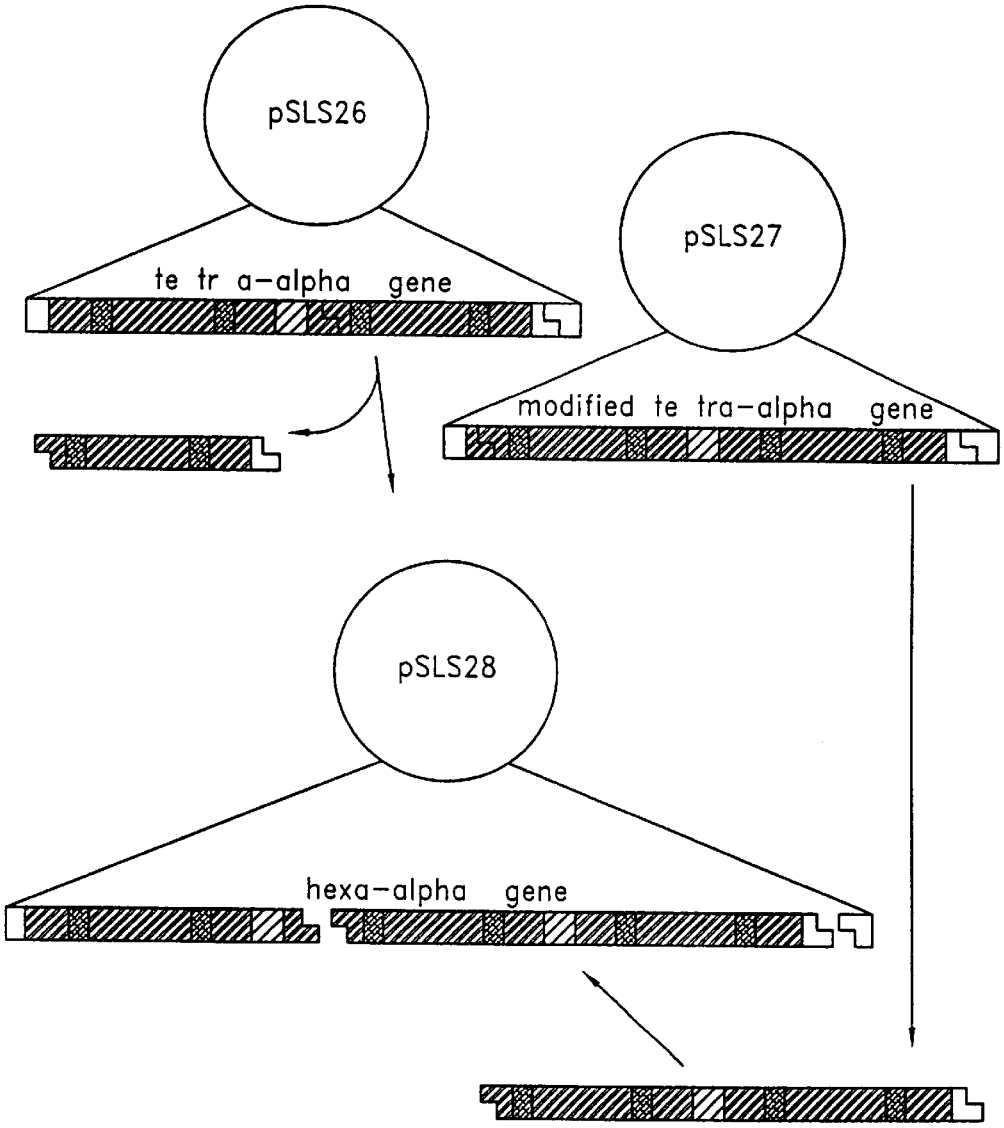

pSLS26
te tr a-alpha gene
pSLS27
modified te tra-alpha gene
pSLS28
hexa-alpha gene

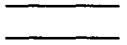
Figure 16

Figure Legends:

All plasmids represented in figures 1,5,6,8,10,11,12,13, and 14 are pUC based plasmids containing the ampicillin resistance gene and the colE1 origin. The genes cloned into the vectors are under the control of the *lac* promoter. The following is a legend for the plasmid schematics:

alpha-globin or alpha -globin like gene or gene fragment

beta-globin gene



non amino acid coding DNA sequence

Restriction enzyme site

Glycine codon used to link 2 alpha-globin genes

DNA coding Gly-Ser-Gly-Gly used to link 2 Circularly permuted di-alpha genes

Figure 17

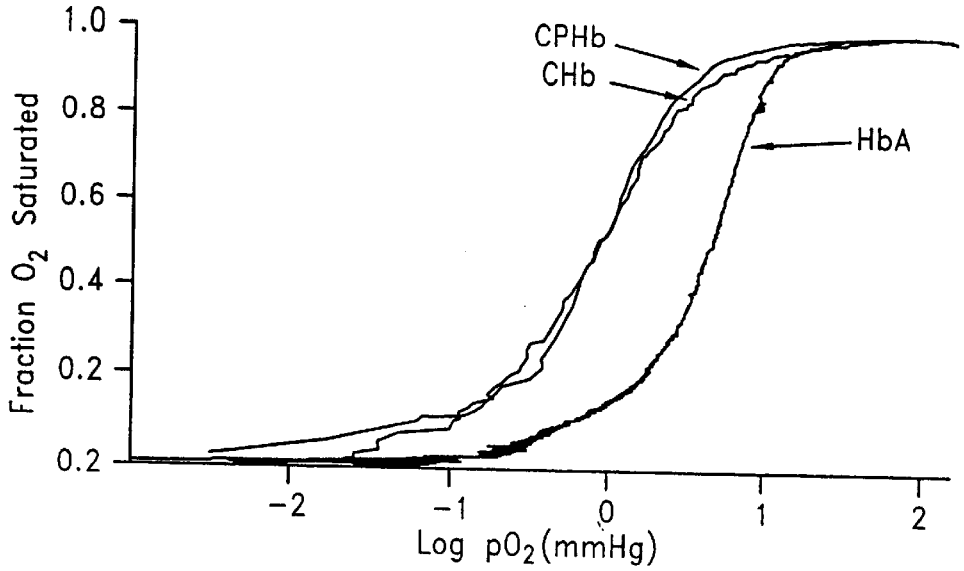
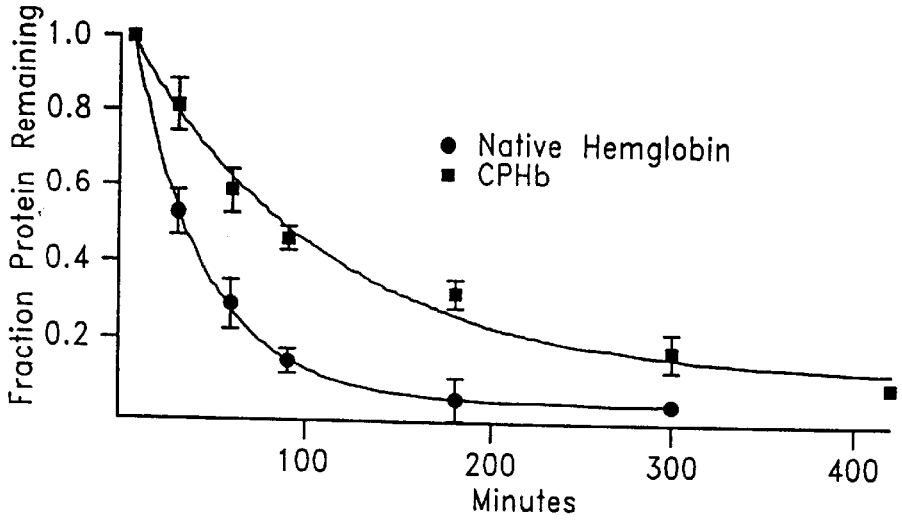


Figure 18



OXYGEN-BINDING HEME PROTEINS INCORPORATING CIRCULARLY-PERMUTED GLOBINS

REFERENCE TO RELATED APPLICATION

This application is a 371 of PCT/US97/17294, filed Sep. 26, 1997.

This application claims priority upon U.S. Provisional patent application Ser. No. 60/026,831 filed Sep. 27, 1996, which is hereby incorporated herein by reference in its entirety.

This invention was made using government support under National Institutes of Health Grant No. PHS 5P01 HL-51084. The Government has certain rights in the invention.

BACKGROUND OF THE INVENTION

The present invention relates generally to oxygen-binding heme proteins, and in particular to such proteins incorporating one or more hemoglobin tetramers incorporating at least one functional, circularly-permuted globin.

As further background, blood transfusions allow trauma patients means to replenish blood loss, surgery patients to enter longer procedures with less risk, and rescue workers to bring a blood supply to accident victims. Although a transfusable blood supply provides many benefits, available blood is limited by human donations. In addition, the limited shelf-life of whole blood, disease transfer, and mismatched blood typing are problems yet to be fully addressed.

For example, the occurrence of an HIV-contaminated blood supply in the 1980's heightened awareness for a need to circumvent the problems associated with a donated blood supply. Even today, the United States Department of Health and Human Services has created a blood safety panel to examine many issues relative to donated blood, including HIV and hepatitis.

Transfused blood, containing plasma, white blood cells (leukocytes), platelets and red blood cells (erythrocytes), is generally used to carry oxygen from the lungs to the rest of the body's cells. A number of oxygen carrying solutions are being studied as alternatives to blood transfusions. In this regard, an effective blood substitute must satisfy three basic requirements. First, it must transport oxygen from lungs to tissues. Second, it must remain functional in vivo long enough to be effective; and third, it must not elicit harmful side effects. Blood substitutes studied to date include perfluorocarbons (Kaufman, R. J. (1991) in *Biotechnology of Blood* (J. Goldstein, e., Ed.) pp. 127–162, Butterworth-Heinemann, Boston), chemically modified hemoglobin from outdated human blood (Winslow, R. M. (1992) *Hemoglobin-based red cell substitutes,* Johns Hopkins University Press, Baltimore), and recombinant hemoglobins produced in microbial and mammalian hosts (Shen, T. -J., Ho, N. T., Simplaceanu, V., Zoiu, M., Green, B. N., Tam, M. F., & Ho, C. (1963), *PNAS USA* 90, 8108–8112; Rao, M. J., Schneider, K., Chait, B. T., Chao, T. L., Keller, H. Anderson, S., Manjula, B. N., Kumar, R., & Acharya, A. S. (1994) *ACBSIB* 22, 695–700).

On the subject of hemoglobin, each hemoglobin molecule is a tetramer of four smaller polypeptide subunits known as globins. A heme group, which is an iron-protoporphyrin complex, is associated with each polypeptide subunit, and is responsible for the reversible binding of a single molecule of oxygen. Normal adult hemoglobin is made up of two different kinds of polypeptide globins. A first globin, known as alpha globin, contains 141 amino acid residues. The second, known as beta globin, contains 146 amino acid residues. In normal adult hemoglobin, two of each kind of globin are arranged in the form of a truncated tetrahedron which has the overall shape of an ellipsoid.

The overall hemoglobin molecule is a 64,400 kDa protein. X-ray crystal structures show the size of HbAo to be about 64 Å×55 Å×50 Å (Fermi, G., Perutz, M. F., Shaanan, B. and Fourme, B. (1984) *Journal of Molecular Biology* 175, 159). The heme prosthetic group of each alpha subunit is non-covalently bound to the subunits by Lys E10, His CD3, Val E11, and Phe CD1. In beta chains, His CD3 is replaced by Ser CD3 The heme contains an Fe++ bound by the proximal histidine. A distal histidine hovers over the iron but does not coordinate; however, this histidine could sterically and/or electronically hinder the binding of CO, which has a higher affinity for heme than O_2, as well as hydrogen bond to iron in the deoxystate. The irons in the hemes can oxidize to the Fe+++ state, creating a nonfunctional hemoglobin (Bunn, H. F. a. F., B. G. (1986) in *Hemoglobin-Molecular, Genetic, and Clinical Aspects* (Dyson, J., Ed.) pp. 13–19, W. B. Saunders Company, Philadelphia).

Ligands that bind hemoglobin include CO, NO, CN—, and the most physiologically relevant ligand, O_2. Oxygen binding occurs in a sygmoidal pattern, demonstrating the cooperativity of multiple ligand binding. It has been shown that hemoglobin can exist in at least two states, T and R. The T state is associated with the deoxygenated state of hemoglobin, while the R state is associated with ligand bound hemoglobin. A number of models have been offered to describe the shift from T to R when ligand is bound. Two primary models describe the change in states as either a concerted change from T to R or a sequential change of subunits from T to R as ligand is bound. The concerted model proposed by Monod, Wynman, and Changeux describes cooperativity resulting from the entire tetramer converting from T to R (Monod, J., Wyman, J., and Changeux, J. -P. (1965) *Journal of Molecular Biology* 12, 88–118). The induced fit mode describes cooperativity as the result of an R state, ligand bound subunit inducing a neighboring T state subunit to alter to the R conformation (Koshland, D. E., Nemethy, G. and Filmer, D. (1966) *Biochemistry* 5, 365–385). Recently, Ackers and co-workers have proposed a symmetry model for T to R transition which provides evidence for an intermediate state in T to R transition (Ackers, G. K., Doyle, M. L., Myers, D., and Daugherty, M. A. (1992) Science 255, 54–63). The eight intermediate ligation states have been studied using metal-substituted hemes that are unable to bind ligand. The evidence demonstrates the steepest free energy change occurs when a subsequent ligand binds the alternate alpha/beta dimer.

Ligand affinity is also dependent on a number of allosteric effectors. The effectors that lower oxygen affinity include protons (Bohr effect), 2,3 diphosphoglycerate, and chloride ions. The physiological relevance of the effectors is to enhance oxygen delivery to metabolically active cells that produce CO_2.

Modification of human hemoglobin has been widely investigated as a means to provide a blood substitute and for other uses. Hemoglobin is a well-characterized protein, and can be altered to meet the basic requirements for an effective and safe blood substitute. Chemically modified, and more recently, recombinant forms of hemoglobin, are currently being tested in various stages of clinical trials.

Some problems arise from overproduction of recombinant hemoglobin in prokaryotes and eukaryotes. In humans,

methionine aminopeptidase recognizes small, hydrogphobic residues as a signal to cleave (Hernan, R. A., Hui, H. L., Andracki, M. E., Noble, R. W., Sligar, S. G., Walder, J. A., & Walder, R. Y. (1992), Biochemistry 31, 8619–8628). Therefore, the first amino acid in postranslationally modified human hemoglobin is a valine. However, during the expression of human hemoglobin in *E. coli,* the initial methionine is not cleaved. Further, *E. coli* methionine peptidase recognizes small polar side chains, and expression in *E. coli* essentially adds a methionine to the primary sequence of both alpha and beta chains. This issue has been dealt with in two ways. A yeast expression system has been utilized in which the initial methionine is cleaved (Wagenbach, M., O'Roueke, K., Vitez, L., Wieczorek, A., Hoffman, S., Durfee, S., Tedesco, J., & Stetler, G. (1991) *Bio-Technology* 9, 57–61). In prokaryotic production, the replacement of the first amino acid - valine - with a methionine was used in both alpha and beta chains (recombinant hemoglobin des-val) to produce a protein functionally similar to HbAo (Hernan, R. A., Hui, H. L., Andracki, M. E., Noble, R. W., Sligar, S. G., Walder, J. A., & Walder, R. Y. (1992), *Biochemistry* 31, 8619–8628).

It has been reported that these overproduced hemoglobins are misassembled in the yeast and *E. coli* (Hernan, R. A., & Sligar, S. G. (1995) *JBC,* 270, 26257–26264). The misassembled tetramer initially binds ligand similarly to wild type hemoglobin, but over time drifts to different tetramer substrates that bind ligand at different rates. The drift appears to be time and temperature dependent, and protein stored at −70° C. still encounters a drift problem. Wild type hemoglobin stored at −70° C. has not demonstrated a similar effect.

Studies have shown that hemoglobin blood substitutes offer a number of difficulties as well as benefits. Hemoglobin is a powerful tool for oxygen delivery, but its use removes a tightly regulated protein from its native environment. One major problem for hemoglobin based blood substitutes occurs when oxygen in the heme iron dissociates as superoxide ion, leaving hemoglobin oxidized in the ferric "met" state. This autoxidation leaves hemoglobin in a state where it cannot bind ligand. Moreover, the Fe+++ state is an intermediate in the pathway to the highly reactive Fe^{4}+ ferryl state, heme loss, and can cause peroxidation of lipids (Giulivi, C., and Davies, K. J. A. (1994), *Methods of Enzymology* 231, 490–496; Yamamoto, Y., and La Mar, G. N. (1986) *Biochemistry* 25, 5288–5297; Repka, T., and Hebbel, R. P. (1991) *Blood* 78, 2753–2758). Superoxide off-rates appear to govern the measured autoxidation rate.

Another key problem associated with hemoglobin-based blood substitutes is hemoglobin's affinity for nitric oxide (NO), which is higher than its affinity for CO or O_2. NO is a vasodilator and can be carried by hemoglobin as a heme ligand or on a cysteine as a nitrosothiol (Bonaventura (1996) *Nature* 380, 221–226). Results in clinical trials demonstrate that patients treated with a hemoglobin-based blood substitute often encounter higher blood pressure (Blantz, R. C., Evan, A. P., and Gabbai, F. B. (1995) in *Blood Substitutes: Physiological Basis of Efficacy* (Winslow, R. M., Vandegriff, K. D., and Intaglietta, M., Ed.) pp. 132–142, Birkhauser, Boston). Another problem with hemoglobin is that the molecule is small enough to extravasate into the endothelial lining and bind NO. Patients treated with L-arginine, an intermediate in the NO synthesis pathway, or nitroglycerine, a vasodilator, have normal blood pressures while being administered hemoglobin solutions (see Blantz, R. C., Evan, A. P., and Gabbai, F. B. (1995) in *Blood Substitutes: Physiological Basis of Efficacy,* supra.

Perhaps the most significant drawback of hemoglobin blood substitutes is the rapid filtration of hemoglobin molecules by the kidney. At concentrations used in patients, hemoglobin dissociates into alpha/beta dimers small enough for renal filtration. This not only significantly decreases the lifetime of the blood substitute (half life of less than an hour), but it also deleteriously effects renal tubules and can cause renal toxicity (see Blantz, R. C., Evan, A. P., and Gabbai, F. B. (1995) in *Blood Substitutes: Physiological Basis of Efficacy,* supra).

One important step in eliminating renal toxicity is the cross-linking of alpha/beta dimers. Current efforts include chemical cross-linking of two alphas or two betas with a covalent attachment to lysine residues (Vandegriff, K. D., & Le Telier, Y. C. (1994) *Artificial-Cells-Blood-Substitutes-and-Immobilization-Biotechnology* 22, 443–455). In addition, hemoglobins have been randomly polymerized using glyceraldehyde (Vandegriff, K. D., & Le Telier, Y. C. (1994) *Artificial-Cells-Blood-Substitutes-and-Immobilization-Biotechnology* 22, 443–455). However, utilization of a chemical reaction significantly lowers the yield of functional protein.

Researchers have produced a genetically cross-linked hemoglobin molecule with a half life of almost two hours (Looker, D., Abbott-Brown, D., Cozart, P., Durfee, S., Hoffman, S. Mathews, A., Miller-Roehrich, J., Shoemaker, S., Trimble, S., Fermi, G., Komiyama, N. H., Nagai, K., & Stetler, G. L. (1992) *Nature* 356, 258–260). X-ray crystallography has shown the C-terminus of one alpha chain to be only 2 to 6 Å away from the N-terminus of the second alpha chain (Shaanan, B., (1983) *Journal of Molecular Biology* 171, 31–59), and trypsin catalyzed reverse hydrolysis has demonstrated that an additional amino acid attached to the C-terminus does not alter oxygen binding properties. These results, coupled with the knowledge that the C-terminal arg141 can form a salt bridge with the alternate alpha chain's vall, demonstrated the feasibility of genetically cross-linking the two alpha chains. The di-alpha chain expressed by these workers in *E. coli* consisted of an alpha des-val, a glycine linker, and a native alpha chain sequence. The construct was co-expressed with a des-val version of a naturally occurring low-oxygen affinity beta mutant (beta Presbyterian, R108K), and the entire construct was dubbed rHb1.1.

Despite these extensive efforts to develop a hemoglobin-based blood substitute, needs still exist for substitutes with increased crosslinking and higher molecular weight, which provide increased molecular stability and plasma half-life, and a decreased risk of renal toxicity. Such substitutes will desirably be readily expressed in host cells in high yield and have advantageous oxygen-binding capacity. The present invention addresses these needs.

SUMMARY OF THE INVENTION

Accordingly, one preferred embodiment of the invention provides a heme protein which includes a (i.e. at least one) hemoglobin molecule including at least one circularly permuted globin. In a preferred form, the invention takes advantage of the close proximity of the N and C termini of neighboring alpha chains, and a linker of one or more amino acids is inserted between both sets of termini. New termini are formed at any sequence position in the protein, and preferably at a position so as to be surface-exposed for linkage with other molecules, for example one or more other hemoglobin molecules to form recombinant hemoglobin multimers. Preferred proteins of the invention include at least one oxygen-binding hemoglobin tetramer having two

alpha and two beta globins, wherein at least one of the globins is circularly permuted, and, more preferably, has surface-exposed N- and C-termini. Still more preferably, the hemoglobin molecule(s) in proteins of the invention will have multiple crosslinks between globins.

Another preferred embodiment of the invention provides a heme protein, preferably oxygen-binding, which includes at least one hemoglobin molecule including two beta globins and a di-alpha globin construct. The di-alpha globin construct includes a circularly-permuted alpha globin genetically crosslinked to another alpha globin. Thus the preferred di-alpha globin construct will include an amino acid sequence corresponding to a circular permutation of single polypeptide which has alpha chains whose original N- and C-termini are each linked to one another by a linker sequence of one or more amino acids. In an advantageous form, the di-alpha construct can be covalently linked to another protein, e.g. another di-alpha construct, by a polypeptide linker, to form proteins of high molecular weight, e.g. hemoglobin multimers.

Another preferred embodiment of the present invention provides a polynucleotide coding for a single polypeptide having a circularly-permuted alpha globin covalently linked to another alpha globin by two genetic crosslinks. Thus, preferred polynucleotides will sequentially encode (1) a first portion of a first, circularly-permuted alpha globin; (2) a first genetic crosslink; (3) a second alpha globin; (4) a second genetic crosslink; and (5) a second portion of the circularly permuted alpha globin, the first and second portions together constituting the entire circularly-permuted alpha globin. Thus, preferred polynucleotides will code for two alpha globins, a first of which is circularly permuted and a second of which is non-circularly permuted and occurs in the polypeptide linking the original N- and C-termini of the first alpha globin.

Still another preferred embodiment of the invention provides a circularly-permuted globin having termini located within a surface-exposed loop region of the globin (i.e. within any non-helicle surface-exposed alpha segment). The preferred, surface-exposed termini will be solvent-exposed (having no structures of the globin overlying the termini), and effective for covalent linking of one or both termini to an adjacent hemoglobin alpha or beta subunit, or to another molecule, e.g. to form a fusion protein. Preferred circularly-permuted alpha globins will have as terminal amino acids, residues 47 and 48, 48 and 49, 49 and 50, 50 and 51, 113 and 114, 114 and 115, 115 and 116, or 116 and 117 of the corresponding non-circularly permuted globin. Preferred circularly-permuted beta globins will have as terminal amino acids, residues 46 and 47, 47 and 48, 48 and 49, 118 and 119, 119 and 120, 120 and 121 and 121 and 122 of the non-circularly permuted beta globin.

Other preferred embodiments of the invention provide a polynucleotide encoding a circularly-permuted globin, a vector or host cell including such a polynucleotide, and a method for preparing a heme protein which involves culturing a host cell including and expressing such a polynucleotide.

The present invention also relates to a method of increasing tissue oxygenation in a warm blooded animal patient, e.g. human patient, comprising administering to the patient a therapeutically effective amount of an oxygen-binding heme protein of the invention.

The present invention also provides a method of replacing hemoglobin in the bloodstream of a warm blooded animal patient, e.g., a human patient, comprising administering to the patient an effective amount of a heme protein of the invention.

A still further preferred embodiment of the invention provides a method for inducing vasoconstriction in a warm blooded animal, e.g. a human patient, comprising introducing into the blood stream of the animal an effective amount of an oxygen-binding heme protein of the invention.

Another preferred embodiment of the invention provides a method for increasing the oxygenation of an isolated organ or tissue, for example during storage or transport, which includes the step of contacting the organ or tissue with an oxygen-binding heme protein of the invention.

Additional embodiments as well as objects, features and advantages of the invention will be apparent from the following description.

DESCRIPTION OF THE FIGURES

FIG. **1** provides a schematic representation of the ligation procedure used to generate pSS1.

FIG. **2** shows the oligonucleotide cassette used in the generation of pSS1 (SEQ ID NOS: 1 and 2).

FIG. **3** shows the DNA sequence of the di-alpha gene contained in pSS1 (SEQ ID NOS: 3 and 5). The resulting amino acid sequence (SEQ ID NO: 4) and primary restriction sites are also shown.

FIG. **4** shows oligonucleotide cassettes, CP1 (SEQ ID NOS: 6 and 7) and CP2, (SEQ ID NOS: 8 and 9) used in the generation of pSLS21 and pSLS22 respectively.

FIG. **5** provides a schematic representation of the ligation procedure used to generate pSLS21.

FIG. **6** provides a schematic representation of the ligation procedure used to generate pSLS22.

FIG. **7** shows the DNA sequence (SEQ ID NOS: 10 and 12) of the circularly permuted di-alpha gene contained in pSLS22. The resulting amino acid sequence (SEQ ID NO: 11) and primary restriction sites are also included. Regions highlighted in bold are the glycine codon linking regions.

FIG. **8** shows schematic representation of the ligation procedure used to generate pSLS23.

FIG. **9** shows oligonucleotide cassettes, TA1 (SEQ ID NOS: 13 and 14) and TA2 (SEQ ID NOS: 15 and 16), used in the generation of pSLS24 and pSLS25 respectively.

FIG. **10** provides a schematic representation of the ligation procedure used to generate pSLS24.

FIG. **11** provides a schematic representation of the ligation procedure used to generate pSLS25.

FIG. **12** provides a schematic representation of the ligation procedure used to generate pSLS26.

FIG. **13** shows the DNA sequence (SEQ ID NOS: 17 and 19) of the tetra-alpha gene contained in pSLS26. The resulting amino acid sequence (SEQ ID NO: 18) and primary restriction sites are also included. Regions highlighted in bold are the single glycine and Gly-Ser-Gly-Gly linking regions.

FIG. **14** provides a schematic representation of the ligation procedure used to generate pSLS27. The modified tetra-alpha gene contains slightly different restriction sites.

FIG. **15** provides a schematic representation of the ligation procedure used to generate pSLS28.

FIG. **16** provides a legend for the plasmid schematics shown in FIGS. **1**, **5**, **6**, **8**, **10**, **11**, **12**, **14** and **15**.

FIG. **17** shows equilibrium binding curves for native hemoglobin (HbA), circularly permuted hemoglobin (CPHb) and octameric hemoglobin (OHb).

FIG. **18** shows the results of plasma lifetime measurement of HbA and CPHb.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

For the purposes of promoting an understanding of the principles of the invention, reference will now be made to embodiments thereof and specific language will be used to describe the same. It will nevertheless be understood that no limitation of the scope of the invention is thereby intended, such alterations, further modifications and applications of the principles of the invention as described herein being contemplated as would normally occur to one skilled in the art to which the invention pertains.

The following definitions are used herein.

Nucleotide—A monomeric unit of DNA or RNA containing a sugar moiety (pentose), a phosphate, and a nitrogenous heterocyclic base. The base is linked to the sugar moiety via the glycosidic carbon (1' carbon of the pentose) and that combination of base and sugar is called a nucleoside. The base characterizes the nucleotide. The four DNA bases are adenine ("A"), guanine ("G"), cytosine ("C"), and thymine ("T"). The four RNA bases are A, G, C and uracil ("U").

Polynucleotide—A linear array of nucleotides connected one to the other by phosphodiester bonds between the **3'** and **5'** carbons of adjacent pentoses.

Polypeptide—A linear array of amino acids connected one to the other by peptide bonds between the alpha-amino and carboxy groups of adjacent amino acids.

Expression—The process undergone by a structural gene to produce a polypeptide. It is a combination of transcription and translation.

Plasmid—A non-chromosomal double-stranded DNA sequence comprising an intact "replicon" such that the plasmid is replicated in a host cell.

Vector—A plasmid, viral DNA or other DNA sequence which is capable of replicating in a host cell, which is characterized by one or a small number of endonuclease recognition sites at which such DNA sequences may be cut in a determinable fashion without attendant loss of an essential biological function of the DNA, e.g., replication, production of coat proteins or loss of promoter or binding sites, and which contains a marker suitable for use in the identification of transformed cells, e.g., tetracycline resistance or ampicillin resistance

Transformation—The introduction of DNA or RNA into cells in such a way as to allow gene expression.

Stroma—free preparation—a preparation free from red blood cells and red blood cell membrane fragments.

Crosslinked Hemoglobin Molecule—A hemoglobin molecule modified by covalent bond crosslinkage between one or more of its globin subunits.

Circularly-Permuted Globin—A globin having a covalent bond linkage between its native terminal amino acid residues and which has new terminal amino acid residues at another location of its polypeptide chain.

Genetic Crosslink—An amino acid or a polypeptide which covalently links two globins of a hemoglobin tetramer and which is formed upon expression of a polynucleotide encoding the two globins and the chain as a single polypeptide.

Native Termini—The termini amino acid residues of a globin prior to its circular permutation.

New Termini—The terminal amino acid residues of a globin after its circular permutation.

Globin—A compact protein domain containing heme preferably capable of forming higher molecular weight aggregates.

Hemoglobin—A protein of four globins.

Circularly-Permuted Hemoglobin Multimer—A protein which includes two or more hemoglobin molecules each having at least one circularly-permuted globin, wherein the circularly-permuted globins of adjacent hemoglobin molecules are covalently linked to one another by a linker chain of one or more amino acids.

As disclosed above, the present invention concerns novel heme proteins which have at least one hemoglobin molecule including at least one circularly-permuted globin. In this regard, a circularly permuted (CP) protein has its native termini linked, and new termini at some other location in its polypeptide chain. Thus, circularly-permuted proteins can be prepared by creating a circular primary sequence of the protein, and then recleaving the protein at another site, or by expression of a DNA sequence encoding an amino acid sequence corresponding to the recleaved protein. The resulting protein represents a new protein which has a primary amino acid sequence which differs significantly from that of the starting protein. However, for the sake of simplicity in nomenclature, the art has adopted the practice of referring to the new protein as a circularly-permuted "starting protein", a practice which will be followed herein for the sake of convenience when referring to a peptide having a primary amino acid sequence which corresponds to a circular permutation of the primary amino acid sequence of a known globin such as an alpha or beta globin. Similarly, tetrameric heme proteins of the invention which incorporate one or more circularly-permuted globins will be referred to as hemoglobins.

The present invention can be applied to conventional human hemoglobin and a wide variety of known hemoglobin mutants. In this regard, the amino acid sequences for the alpha and beta globins of conventional human hemoglobin are provided in Table 2, in which the abbreviations in Table 1 are employed.

TABLE 1

Amino Acid	Abbreviation
Alanine	Ala
Arginine	Arg
Asparagine	Asn
Aspartic acid	Asp
Cysteine	Cys
Glutamine	Gln
Glutamic acid	Glu
Glycine	Gly
Histidine	His
Isoleucine	Ile
Leucine	Leu
Lysine	Lys
Methionine	Met
Phenylalanine	Phe
Proline	Pro
Serine	Ser
Threonine	Thr
Tryptophan	Trp
Tyrosine	Tyr
Valine	Val

TABLE 2

Beta Globin		Alpha Globin	
1	Val	1	Val
2	His	2	Leu
3	Leu	3	Ser
4	Thr	4	Pro

TABLE 2-continued

Beta Globin		Alpha Globin	
5	Pro	5	Ala
6	Glu	6	Asp
7	Glu	7	Lys
8	Lys	8	Thr
9	Ser	9	Asn
10	Ala	10	Val
11	Val	11	Lys
12	Thr	12	Ala
13	Ala	13	Ala
14	Leu	14	Trp
15	Trp	15	Gly
16	Gly	16	Lys
17	Lys	17	Val
18	Val	18	Gly
19	Asn	19	Ala
20	Val	20	His
21	Asp	21	Ala
22	Glu	22	Gly
23	Val	23	Glu
24	Gly	24	Tyr
25	Gly	25	Gly
26	Glu	26	Ala
27	Ala	27	Glu
28	Leu	28	Ala
29	Gly	29	Leu
30	Arg	30	Glu
31	Leu	31	Arg
32	Leu	32	Met
33	Val	33	Phe
34	Val	34	Leu
35	Tyr	35	Ser
36	Pro	36	Phe
37	Trp	37	Pro
38	Thr	38	Thr
39	Gln	39	Thr
40	Arg	40	Lys
41	Phe	41	Thr
42	Phe	42	Tyr
43	Glu	43	Phe
44	Ser	44	Pro
45	Phe	45	His
46	Gly	46	Phe
47	Asp	47	Asp
48	Leu	48	Leu
49	Ser	49	Ser
50	Thr	50	His
51	Pro	51	Gly
52	Asp	52	Ser
53	Ala	53	Ala
54	Val	54	Gln
55	Met	55	Val
56	Gly	56	Lys
57	Asn	57	Gly
58	Pro	58	His
59	Lys	59	Gly
60	Val	60	Lys
61	Lys	61	Lys
62	Ala	62	Val
63	His	63	Ala
64	Gly	64	Asp
65	Lys	65	Ala
66	Lys	66	Leu
67	Val	67	Thr
68	Leu	68	Asn
69	Gly	69	Ala
70	Ala	70	Val
71	Phe	71	Ala
72	Ser	72	His
73	Asp	73	Val
74	Gly	74	Asp
75	Leu	75	Asp
76	Ala	76	Met
77	His	77	Pro
78	Leu	78	Asn
79	Asp	79	Ala
80	Asn	80	Leu
81	Leu	81	Ser

TABLE 2-continued

Beta Globin		Alpha Globin	
82	Lys	82	Ala
83	Gly	83	Leu
84	Thr	84	Ser
85	Phe	85	Asp
86	Ala	86	Leu
87	Thr	87	His
88	Leu	88	Ala
89	Ser	89	His
90	Glu	90	Lys
91	Leu	91	Leu
92	His	92	Arg
93	Cys	93	Val
94	Asp	94	Asp
95	Lys	95	Pro
96	Leu	96	Val
97	His	97	Asn
98	Val	98	Phe
99	Asp	99	Lys
100	Pro	100	Leu
101	Glu	101	Leu
102	Asn	102	Ser
103	Phe	103	His
104	Arg	104	Cys
105	Leu	105	Leu
106	Leu	106	Leu
107	Gly	107	Val
108	Asn	108	Thr
109	Val	109	Leu
110	Leu	110	Ala
111	Val	111	Ala
112	Cys	112	His
113	Val	113	Leu
114	Leu	114	Pro
115	Ala	115	Ala
116	His	116	Glu
117	His	117	Phe
118	Phe	118	Thr
119	Gly	119	Pro
120	Lys	120	Ala
121	Glu	121	Val
122	Phe	122	His
123	Thr	123	Ala
124	Pro	124	Ser
125	Pro	125	Leu
126	Val	126	Asp
127	Gln	127	Lys
128	Ala	128	Phe
129	Ala	129	Leu
130	Tyr	130	Ala
131	Gln	131	Ser
132	Lys	132	Val
133	Val	133	Ser
134	Val	134	Thr
135	Ala	135	Val
136	Gly	136	Leu
137	Val	137	Thr
138	Ala	138	Ser
139	Asn	139	Lys
140	Ala	140	Tyr
141	Leu	141	Arg
142	Ala		
143	His		
144	Lys		
145	Tyr		
146	His		

There are also hundreds of known mutations of hemoglobin which involve changes in the amino acid structure of the polypeptide chains. For example, a mutant form of alpha globin (des-val) is used in the specific Experimental below. This alpha globin has a valine→methionine substitution at amino acid 1 of the chain. Other known alpha mutants include but are not limited to such modifications as amino acid 94 aspartic acid →asparigine in the alpha chain (Hb Titusville).

A number of known mutant hemoglobins have amino acid substitutions at human beta globin positions 90, 102, 108 and combinations thereof. Some specific examples of beta mutations are but are not limited to:

(1) amino acid 90 glutamine→lysine (hemoglobin Agenogi)

(2) amino acid 90 glutamine→glycine

(3) amino acid 108 asparagine→aspartic acid (hemoglobin Yoshizuka)

(4) amino acid 102 asparagine→threonine (hemoglobin Kansas)

(5) amino acid 102 asparagine→serine (hemoglobin Beth Israel)

(6) amino acid 90 glutamic acid→valine amino acid 91 Leucine→methionine amino acid 93 cysteine→serine amino acid 94 aspartic→glutamic acid

A Table including a listing of some additional illustrative hemoglobin variants is set forth in Appendix A attached hereto and made a part hereof, taken from *Hemoglobin,* Vol 19, No. 1–2, pp. 39–124, Marcel Dekker (1995).

In addition to known mutations other mutations can be engineered into these circularly permuted globins in order to add additional desirable properties into the protein or protein multimer. For example, mutations that alter the electronic environment of the heme may be included to stabilize the reduced, physiologically active, form of the molecule or alter the ligand affinity and selectivity.

Generally speaking, in the present invention, the new termini of the globin subunit are formed at a site that does not eliminate the function of the globin in assembling with other globins to form an oxygen-binding, tetrameric heme protein. Thus, the resulting protein will possess the function of interest of the wild type hemoglobin, e.g. the capacity to bind oxygen at some level, which can be the same level, or a level which is increased or decreased relative to the wild type protein.

Generally speaking, preferred candidate locations for forming the new termini will fall within surface-exposed loop regions on the globins, rather than in alpha helical segments. This is expected to minimize disruption of the protein structure since the loops are not highly ordered. More preferred regions for introducing new termini in alpha globins include the loop region between the C and E helices (residues 47–51) and the loop region between the G and H helices (residues 113–117). The loop region between the C and E helices is most preferred. Thus, in the Experimental below, new termini were created at original serine 49 (new N terminus) and original leucine 48 (new C terminus) of normal adult human alpha globin.

In the circular permutation of a human beta globin (see e.g. Table 1), a longer linker may be used to join the native termini because the termini are not as spacially close as those of the alpha chains. For example, a polypeptide linker of about three to five residues may be used. Because the beta chains are structurally similar to the alpha chains, the preferred sites for introduction of new termini in beta chains generally include the same loop regions selected for the alpha chains. These include, but are not limited to, the loop region between helices C and D (residues 46–49), and the loop region between helices G and H (residues 118–122). Of these, the loop region between helices C and D is most preferred.

The new termini of the circularly-permuted globins of the invention are preferably exposed on the surface of the globin when assembled in the ellipsoid, tetrameric hemoglobin. The selection of new termini may be assisted in this regard by conventional modeling software, for example modeling protein structure on a Silicon Graphics Imaging computer using molecular modeling software to verify surface exposure of amino acids. Location of the new termini at the surface of the hemoglobin molecule facilitates covalent linkage of the molecule to other molecules through amino acid or polypeptide linkers. In one preferred practice of the invention, a hemoglobin multimer is provided, in which a plurality of hemoglobin molecules are covalently linked to one another by polypeptide linkers spanning between circularly-permuted globins of the respective hemoglobin molecules. In this regard, the length of this polypeptide linker can vary widely to suit a particular application; however, it is expected that polypeptide linkers having about one to about twenty amino acids will be suitable for most applications, more commonly having about one to about ten amino acids. In the applicants' preferred work, the polypeptide linker included a number of glycine residues in order to impart conformational freedom to the linker. In addition, the intermolecular linker will likely be solvent exposed, and thus hydrophilic residues can be used to advantage, for example, amino acid residues containing hydroxyl or acidic groups, e.g. the hydroxyl-containing serine used in the specific work reported in the Experimental below. Generally speaking, the selection and use of suitable amino acids in the intermolecular linker will be well within the purview of those skilled in the field.

Similarly, the number of hemoglobin molecules in hemoglobin multimers of the invention may vary, including multimers having up to and exceeding about one hundred hemoglobin repeating units. Again, for most applications it is expected that a smaller number of repeating units will be suitable, e.g. in the range of two to about ten hemoglobin repeating units.

To create intramolecular crosslinks between globins of a hemoglobin molecule, it is desirable to use a chain of one to about seven amino acids, more preferably from one to about three amino acids. Any suitable amino acid or set of amino acids may be used for this purpose, including for example one or more amino acids selected from those identified in Table 1, above. The selection and use of suitable amino acids in the crosslinks will be well within the purview of those skilled in the field.

Amino acid crosslinks such as those discussed above are conveniently introduced as genetic crosslinks. Other modes of introducing intramolecular and/or intermolecular crosslinks may also be used, including for example chemical treatment with crosslinking agents. Such crosslinking agents may include dialdehydes, such as glyoxal, amlonic dialdehyde, succinic dialdehyde, glutaraldehyde, adipaldehyde, 3-methylglutaraldehyde, propyladipaldehyde, phthalic dialdehyde, terephthaldehyde and malonic dialdehyde. See, e.g. Bonsen et al., U.S. Pat. Nos. 4,001,200; 4,001,401; and 4,053,590; and Bonhard et al., U.S. Pat. Nos. 4,136,093 and 4,336,248.

Preferred crosslinked hemoglobin molecules will exhibit increased molecular stability as compared to native, non-crosslinked hemoglobins. This stability may be demonstrated, for instance, by increased thermal stability (e.g. melting points) of the hemoglobin molecules as compared to their non-crosslinked counterparts.

The present invention also concerns an isolated polynucleotide, preferably DNA sequence, coding for a circularly-permuted globin, such as a human alpha or human beta globin. Such polynucleotides can be created, for example, by chemical synthesis of a polynucleotide having the desired sequence corresponding to a circular permutation

of the globin gene at hand. Such polynucleotides of the invention may also be prepared by ligating the ends of the globin coding sequence of interest, directly or via a base sequence coding for an amino acid linker, and then cleaving the resulting circular sequence to result in the circularly-permuted sequence Genetic manipulations to create circularly-permuted sequences can be applied without undue experimentation to form a wide variety of polynucleotides coding for circularly-permuted globins and constructs including them, in accordance with the present invention.

In a preferred aspect the invention provides a polynucleotide, such as a DNA or RNA sequence, preferably a DNA sequence, which encodes a single polypeptide which includes, in sequence: (I) a first portion of a first, circularly-permuted globin; (ii) a first genetic crosslink; (iii) a second, entire globin; (iv) a second genetic crosslink; and (v) a second portion of the circularly-permuted globin, wherein the first and second portions together constitute the entire circularly-permuted globin. The coded polypeptides also form a part of the present invention and, generally speaking, include a first, circularly-permuted globin having its original N- and C-termini joined by a linking polypeptide including the amino acid sequence of a second, non-circularly-permuted alpha globin. More preferably, the first and second globins of such constructs are alpha globins, and the first and second genetic crosslinks (the peptide sequences occurring between the N- and C-termini of the circularly permuted globin and those of the non-circularly-permuted globin) will have from one to about three amino acids.

DNA or other polynucleotides for use in carrying out the present invention may be synthetically created, by hand or with automated apparatus. Means for synthetic creation of the polynucleotide sequences of the invention are generally known to those of ordinary skill in the art, particularly in light of the teachings contained herein. For additional details as to polynucleotide synthesis, reference can be made to standard texts on the subject including for instance Maniatis et al., *Molecular Cloning- A Laboratory Manual,* Cold Spring Harbor Laboratory (1984), and Horvath et al. *An Automated DNA Synthesizer Employing Deoxynucleoside 3'-Phosphoramidites,* Methods in Enzymology 154:313–326, 1987, both hereby incorporated herein by reference. Additionally, polynucleotide sequences of the invention may be constructed by isolating and modifying a polynucleotide which occurs in nature. For instance, a starting globin polynucleotide may be a restriction fragment isolated from a genomic or cDNA library. The starting polynucleotide can then be manipulated using known techniques to produce a polynucleotide of the invention which encodes a circularly-permuted globin, generally as discussed above.

The invention also provides expression or cloning vectors including polynucleotide sequences of the invention, including for instance plasmid vectors, viral vectors, and the like. The synthesis and/or isolation of necessary and desired component parts of expression or cloning vectors, and their assembly, is within the abilities of those of ordinary skill in the art and, as such, are capable of being performed without undue experimentation.

The present invention also concerns a host cell including a polynucleotide of the invention and which expresses the polynucleotide. Such host cells can be made by transforming a cell with a suitable vector carrying a polynucleotide of the invention, for example a plasmid or viral vector. The polynucleotide of the invention can also be introduced into cells using other known techniques, including for example microinjection, electroporation or the like.

The host cell can be selected from a variety of host cells which effectively express hemoglobin, including for instance mammalian cells such as human, murine or porcine cells, gram positive or negative bacterial cells such as *E. Coli.,* Bacillus or Salmonella, yeast cells such as *Sacharomyces Cerevisiae* or *Sacharomyces Pombe,* or insect cells. Further, host cells which express polynucleotides of the invention can be cultured so as to produce circularly-permuted globins of the invention in high yield. The globins can then be individually isolated or, more preferably, the circularly-permuted globin is co-expressed in the host cell with other globins as necessary to produce the oxygen-binding heme protein including at least one assembled hemoglobin tetramer in the cell. Thus, for instance, in the Experimental below, the di-alpha or tetra-alpha globin constructs were co-expressed in host cells with beta globin, and the corresponding assembled hemoglobin tetramer or octamer were isolated from the cells in high yield. In this regard, isolation and purification of inventive proteins from the cultured host cells can be achieved using conventional techniques such as filtration, centrifugation, chromatography, and the like. Substantially purified preparations of heme proteins of the invention can thereby be prepared.

Heme proteins of the invention exhibit useful properties as blood and hemoglobin substitutes. For example, the tetrameric and octameric heme proteins disclosed in the Experimental exhibit increased stability against thermal denaturation as compared to prior-known hemoglobin-based blood substitutes. Also, ligand binding experiments have demonstrated that these proteins possess ligand binding properties characteristic of wild-type hemoglobin, including oxygen binding, geminate recombination and CO on-rate.

For use, heme proteins can be incorporated into pharmaceutically acceptable carriers to form pharmaceutical compositions, if desired. Sterile, liquid carriers, particularly aqueous carriers, or liposomes or other polymerizing and encapsulating polymers, will be preferred, for example a balanced electrolyte and buffer solution. The heme protein is desirably at a concentration of about 1 to about 20% in solution, with the precise concentration employed depending upon the application. The hemoglobin may also be dissolved in known plasma expanders such as colloids (plasma, albumin) or crystalloids (saline, glucose, dextran, gelatins, Hemasol* or Lactated Ringer's), or contained in natural red blood cells or in artificial red blood cells such as liposomes.

The thus-prepared pharmaceutical preparation can then be conventionally administered to a human or other animal patient, for example by injection, catheterization, or the like. For convenience in these purposes, the pharmaceutical preparation can be contained in a sterile, medical-grade container such as a vial, syringe, or infusion bag.

The oxygen-binding heme proteins of the invention may be used, for instance, in a stroma-free hemoglobin-type blood replacement, or to improve tissue oxygenation in disease states associated with compromised oxygen delivery to tissue including myocardial infarction, stroke, small vessel disease such as diabetes, etc. Heme proteins of the invention can further be used to increase oxygenation of tissues (e.g. tumor cells or other tissues having hypoxic cells due to damage by physical or chemical means, e.g., burns, exposure to chemicals, physical injuries or ionizing radiation). In one specific application, heme proteins of the invention may be used to increase oxygenation in hypoxic tumor cells to be subjected to radiation therapy, so as to increase the efficacy of the therapy (see, e.g., U.S. Pat. No.

5,295,944). Many tumors exhibit oxygen heterogeneity, including regions of hypoxia, which protect tumor cells against the cytotoxic action of ionizing radiation. Examples include solid tumors such as sarcomas, carcinomas and lymphomas, and some cases of dispersed tumor cells wherein masses of tumor cells form which can produce regions of oxygen heterogeneity, e.g. advanced leukemia. In such cases an increase in the oxygenation of the tumor tissue can enhance the effect of radiation therapy on the tissue.

In order to increase oxygen transport to the site of a tumor, a preparation including a heme binding protein of the invention can be administered, e.g. intravenously, to the patient. The chemotherapeutic agent can then be administered, with the amount of time between the administration of the heme protein preparation and chemotherapeutic agent depending upon factors such as the amount of time it takes the heme protein preparation to be fully incorporated into the circulatory system of the host, the lifetime of the preparation, etc. Also, the patient may breath oxygen-enriched gas prior to and after the administration of the ionizing radiation. This can be done by having the host breath oxygen-enriched air, 100% oxygen or carbogen (95% oxygen/5% CO_2), or in certain cases exposing the host to hyperbaric oxygen conditions.

Any type of ionizing radiation which exhibits an antitumor effect can be employed, including as examples X-rays, gamma rays, high-energy electrons and High LET radiation, such as protons, neurons and alpha particles. Such ionizing radiation can be administered using techniques well-known to those skilled in the art. For example, X-rays and gamma rays are applied by external and/or interstitial means from linear accelerators or radioactive sources. High energy electrons can be produced by linear accelerators. High LET radiation is also produced by linear accelerators and can also be applied from radioactive sources implanted interstitially. Dosages of the ionizing radiation are generally those conventionally applied in radiotherapeutic treatment of tumors, although in certain cases usage of the oxygen-binding heme protein may lower the necessary dosage of ionizing radiation.

In another area, heme proteins of the invention can be used in low doses to increase perfusion when desired. For example, the heme protein may be administered to increase blood pressure from abnormally low levels, as in shock of hemorrhagic, cardiogenic or septic origin, or to increase blood pressure from normal levels to effect improved perfusion, for instance as in stroke therapy. Heme proteins of the invention may also be used in oxygen sensors.

In addition, heme proteins of the invention may be attached, e.g. covalently bonded, to other molecules via a surface-exposed, terminal amino acid or a polypeptide extending therefrom, to form additional materials in accordance with the present invention. For example, in one aspect, a heme protein of the invention can be conjugated to another molecule to form an active conjugate with increased vascular retention time as compared to that of the other molecule, and thus effectively modulate (increase) the vascular retention time of the other molecule. In one mode, the conjugate can be prepared by genetically linking a heme protein of the invention with the other molecule. Thus, a polynucleotide coding for both the heme protein and the other molecule can be constructed and introduced into a suitable host cell for expression. Expression of the DNA will then provide the conjugate. Heme proteins of the invention having surface-exposed termini will be particularly advantageous for these purposes. Because the termini are surface-exposed and not integrally involved in the structure of the heme protein, the linkage to other molecules will not significantly disrupt the structure of the heme protein, thus leaving it functional, and will also allow the attached molecule to remain in solution as opposed to being buried within the heme protein. There are a number of therapeutic peptides currently in use which would be expected to benefit from increased retention times when conjugated with heme proteins of the invention, including for instance insulin, erythropoietin, and growth hormones such as somatotropins. In the case of erythropoietin, as an example, the administration of a heme protein of the invention genetically linked to erythropoietin (a hormone which promotes red blood cell production in the body), may be used to replenish red blood cell supply of a patient before the blood substitute has degraded and been filtered by the blood stream.

Heme proteins of the invention can also be attached ex-vivo to non-peptides such as organic molecules, DNA and the like. Numerous methods are known for covalently linking compounds to specific chemical moieties on proteins, such as directing crosslinking reagents to lysine on heme proteins of the invention provide a unique reactive sites on the protein for crosslinking to other molecules, which can be capitalized upon using known chemistries. Illustrative attachment chemistries are described for example in T. E. Creighton (1983) "Proteins: Structure and Molecular Properties", W. H. Freeman, N.Y.; and W. D. Dandliker and A. J. Portman (1971) "Excited States of Proteins and Nucleic Acids", R. F. Steiner and I. Weinryb eds., Plenum Press, N.Y., pp. 199–276.

Heme proteins of the invention can also be attached to pharmaceutically active compounds in a specific 1:1 stoichiometry and it is expected that such will be accomplished without deleterious effect on the structural integrity of the heme proteins. Proteins of the invention may also be attached to targeting agents such as antibodies, or can be used advantageously as MRI imaging agents themselves or attached to other imaging (e.g. MRI or X-ray) or therapeutic agents.

For the purposes of promoting a further understanding of the present invention, its principles and its advantages, the following Experimental is provided. It will be understood that this Experimental is illustrative, and not limiting, of the invention.

EXPERIMENTAL

E. coli strains and plasmid vectors used

The *E. coli* strain DH5a was used in all the genetic engineering and protein expression of the gene constructs described below. Plasmids pHS471 and pWHS486, used in the genetic constructions, were generated previously by Hernan et. al. (Biochemistry, 31:8619–28 (1992)). pUC18 is a commercially available plasmid from New England BioLabs.

Generation of DNA cassettes for genetic engineering

Most of the genetic manipulations utilized cassette mutagenisis, which entails the generation of a pair of complementary oligonucleotides which contain the desired coding region. All oligonucleotides were synthetically prepared by and purchased from the genetic engineering facility at the University of Illinois. 400 picomoles of each oligonucleotide was phosphorylated with 10 units of T4 polynucleotide kinase in 100 μL containing 50 mM Tris-HCl, pH 7.6/10 mM $MgCl_2$/5 mM DTT/100 μM EDTA for 60 min. at 37° C. After phosphorylation, the complementary oligonucleotides were annealled by mixing and heating to 95° C. for 5 min. and allowed to slowly cool to room temperature over a 3–4 hr. period. The resulting fragments have sticky ends corresponding to restriction sites allowing for ligation

to gene fragments or plasmid vectors. For the purposes herein and as generally understood in the art, a "cassette" refers to a pair of complementary oligonucleotides prepared in the fashion described above.

Plasmid Mini-Preparations

All plasmid isolations were performed from a 5 mL overnight culture of *E. coli* DH5a, harboring the plasmid of interest, grown in LB media (10 g Tryptone, 5 g Yeast Extract, 5 g NaCl per liter). The commercially available Qiagen Spin Plasmid Mini-Preparation kit was used to purify the DNA. The Qiagen kit gives a high yield of RNA free plasmid. DNA were eluted in 25 μL of water.

Restriction digests

All restriction digests were performed at 37° C. 1–10 units of enzyme were used for each digestion and the reactions were carried out in a final volume of 10 μL. The enzymes were purchased from New England Bio-Labs or GIBCO BRL. The buffer systems used were supplied with the enzymes.

DNA fragment isolation and purification

Restriction enzyme digested DNA fragments that were subsequently used in ligation reactions were separated on a 1% agarose gel with 10 μg/mL ethidium bromide at 130 V for about 1 hour. The selected DNA bands were removed with a razor blade, and the resulting DNA was isolated from the gel fragment using the GeneClean II kit from BioLab 101.

DNA Ligations

Genecleaned plasmid fragments and oligonucleotides were ligated in a total reaction volume of 20 μL with 1 unit of T4 DNA ligase(GIBCO BRL). Vector to fragment/cassette insert ratios varied between 1:3 and 1:50. Ligations ran for one hour at room temperature or 16° C. overnight.

Competent Cells and Transformations

E. coli DH5a was grown in a 5 mL overnight culture of LB media. 500 μL of the overnight culture was used to inoculate 50 μL of LB. The cells grew 3–4 hours and were spun at 5000 g for 5 minutes. The cells were resuspended in 25 mL of cold 0.1 M $CaCl_2$. After a 20 minute incubation on ice, the cells were again spun down under the same conditions. The pellet was then resuspended in 4 mL of cold $CaCl_2$.

Competent *E. coli* were transformed with finished ligation reactions or mini-prepped DNA. The ligation mixture or 0.5 μg of mini-prepped DNA were mixed with 200 μL of competent cells and incubated on ice for 20 minutes. The cells were heat shocked at 37° C. for 2 minutes and placed immediately on ice for 5 minutes. One mL of LB media was added, and the cells were incubated for one hour at 37° C. in order to produce ampicillin resistance. 200 μL of cells were plated on LB plates with agarose (15 g/L) and ampicillin (0.2 g/L). The remaining cell mixtures were spun down, part of the supernatant was removed, and the pelleted cells were resuspended and plated as well.

DNA Sequencing

Gene sequencing was performed at the University of Illinois DNA Sequencing Facility using a Perkin/Elmer DNA Sequencer with PCR amplification. Sequencing reactions were stopped with fluorescently labeled dideoxy nucleotides. Universal and reverse primers for pUC sequencing initiated the reactions.

Construction of Di-alpha globin gene

The alpha des-val gene (pHS471) was used to create a tandem fusion of two alpha globin gene sequences. pHS471 was digested with SalI and PstI to yield a vector containing the majority of the first alpha globin. Then an alpha gene fragment was generated from the digestion of pHS471 with SacI and PstI. This fragment was then ligated into the vector along with a linking cassette which codes for the last portion of the first alpha gene beginning with the SalI restriction site, a glycine codon, and the first portion of the second alpha gene through the SacI restriction site to generate pSS1. A schematic of this ligation procedure is included in FIG. **1**. The DNA sequence of the linking cassette and the di-alpha gene in pSS1 is in FIGS. **2** and **3**, respectively.

The sequence of the construct was confirmed. The linking region was sub-cloned by digestion of the dialpha vector with BamHI and subcloning into the BamHI site of pUC18. The resulting plasmid was sequenced at the University of Illinois sequencing facility.

Construction of the circularly permuted di-alpha gene

The pSS1 created as described above was utilized for the generation of a circularly permuted dialpha globin gene. Two ligation steps and two cassettes were required for the synthesis. The sequence of cassette CP1 and CP2 are shown in FIG. **4**. In the first step, cassette CP1 and a 400 base pair fragment generated from an MluI/BamHI digest of pSS1 was ligated into pHS471 digested with XbaI and BamHI to generate pSLS21 (FIG. **5**). Then cassette CP2 and a 400 base pair fragment generated from an XhoI/BamHI digest of PSS1 was ligated into pSS21 digested with BamHI and PstI to generate pSLS22 (FIG. **6**).

The DNA sequence of pSLS21 was confirmed by sequencing in both the forward and reverse directions at the University of Illinois sequencing facility. The sequence along with the corresponding amino acids are shown in FIG. **7**.

A single operon was then created consisting of both circularly permuted di-alpha and desVal beta globin gene under the control of a single lac promoter. The circularly permuted di-alpha globin gene, isolated by digestion of pSLS22 with XbaI and PstI, and the beta globin gene, isolated by digestion of pWHS486 with PstI and HindIII, were ligated into pUC18 digested with XbaI and HindIII to generate pSLS23 (FIG. **8**).

Construction of the tetra-alpha gene fusion

Two circularly permuted di-alpha genes were fused with a linking region to create a tetra-alpha gene. This construct when expressed with beta globin genes forms a functionally octomeric globin consisting of 1 tetra-alpha globin and 4 beta globin genes. Three ligation steps and 2 cassettes were required for the creation of the tetra-alpha, beta globin gene operon. Cassettes used in the construction TA1 and TA2 are shown in FIG. **9**. First, the StyI/BamHI fragment of pSLS22 and cassette TA1 was ligated into pSLS22 digested with XbaI and BamHI in order to destroy the StyI site of pSLS22, generating pSLS24(FIG. **10**). Secondly, a 400 b.p. BamHI/XhoI fragment of pSLS22 and cassette TA2 was ligated into pSLS24 digested with BamHI and PstI generating pSLS25 (FIG. **11**). Finally, the XbaI/StyI fragment of pSLS25, the StyI/PstI fragment of pSLS22, and the PstI/HindIII fragment of pWHS486 was ligated into pUC18 digested with XbaI and HindIII generating pSLS26(FIG. **12**). The DNA sequence which was confirmed by sequencing is listed in FIG. **13**. The gene product of this construct will be hereafter referred to as octomeric circularly permuted hemoglobin.

Construction of higher order circularly permuted alpha gene fusions

To generate 6 fused alpha chain genes, 2 ligation steps are required as well as the utilization of a partial digest. First, pSLS26 is digested fully with PstI. The resulting linear DNA vector is then partially digested with BamHI using 3 units of enzyme in a non-ideal buffer for 15 minutes. The 1350 b.p. fragment from this reaction is then isolated and ligated into

pSLS22 digested with BamHI and PstI to generate pSLS27 (FIG. **14**). Then pSLS27 is digested fully with PstI and partially digested, in a similar manner to the previous partial digest, with StyI. The 1500 b.p. fragment from this reaction is ligated into pSLS26 digested with StyI and PstI generating pSLS28(FIG. **15**). In a similar manner any number of higher order alpha gene fusions can be created by the sequential ligation of circularly permuted dialpha segments from StyI partial and PstI digests of pSLS27 into progressively larger alpha gene fusion constructs.

Expression of genetically engineered, novel heme proteins

Plasmids pSLS23 and pSLS26 were transformed into competent *E. coli* DH5a cells. The cultures were grown in 1 L of 2XYT media (16 g tryptone, 10 g yeast extract, and 5 g NaCl/L)containing 200 μg/mL ampicillin and 0.5 mM d-aminolevulinic acid in 6 L shake flasks at 37° C. After 36–48 hours, the cells were harvested by centrifugation at 8000 g for 5 minutes and the cell paste was removed and stored frozen at −70° C.

Whole cell CO difference spectra

To assay protein expression, a Carbon Monoxide (CO) difference spectrum was taken of the *E. coli* cell cultures before protein purification. Cells were grown to stationary phase in the conditions described for protein expression above. A few grains of dithionite were added to 3–5 mL of the culture, and a baseline was recorded. CO was then bubbled through the culture and the spectrum was recorded on a Cary13 spectrophotometer.

Isolation and purification of protein

Cell paste was allowed to thaw over a stream of CO, and all buffers used during the lysis and purification procedure were saturated with CO. Thawed cells were resuspended in 5 times (w/v) 10 mM NaH_2PO_4, pH 6.0, 1 mM EDTA. Cells were lysed by 3–4 passes through a Stansted AO-116 cell disrupter. After cell lysis 80units/mL DNase and 8 units/mL RNase were added to the mixture and allowed to incubate at room temperature for 1 hr. The mixture was then centrifuged at 100,000 g in a Beckman L8-M ultracentrifuge. The supernatant was retained and the pH adjusted to 6.0 with 20 mM NaH_2PO_4. The supernatant was then loaded onto a carboxy methyl cellulose column (Whatman) equilibrated with 10 mM NaH_2PO_4, pH 6.0, 1 mM EDTA. The column was then washed with 4 column volumes of 10 mM NaH_2PO_4, pH 6.0, 1 mM EDTA, and finally eluted with a step gradient to 20 mM Tris-HCl, pH 7.0, 1 mM EDTA. The protein was then collected and concentrated using a PM-30 amicon membrane under nitrogen pressure. The concentrated protein was then flash frozen in liquid nitrogen and stored at −70° C. until used.

Mass spectrometry

Purified protein samples were run over a sephadex G-25 column to exchange the protein into water. Electrospray mass spectrometry was performed on the protein samples at the University of Illinois Mass Spectrometry Facility. The samples were diluted to a concentration of 10 pmol/μL into a 50:50 acetonitrile:water solution containing 0.1% formic acid for the experiments.

The experimentally measured masses of the circularly permuted hemoglobin (CpHb) and octameric circularly permuted hemoglobin (OHb) were compared with calculated values. The beta globins of each protein was measured to be 15900±2 Daltons. This is in good agreement with the calculated value of 15901.4 Daltons. The circularly permuted alpha globin construct of each protein are listed below. However, the calculated values are those expected for cleavage of the initial methionine residue. The values are in good agreement indicating that the proteins do not undergo any post-translational modification, except for initial methionine cleavage, in the bacterial host.

	Calculated	Measured
CpHb di-a	30351.6 Da	30352.6 ± 3.9 Da
OHb tetra-a	60943.6 Da	60940.4 ± 9.2 Da

UV-Vos spectroscopy

Protein samples were reduced by the addition of a few grains of dithionite and exchanged into air saturated water. The oxygen bound spectrum was taken in a Hitachi U-3300 spectrophotometer. Then a few grains of dithionite were added to the sample and a deoxy spectrum was taken in a septa sealed cuvette. Finally, the CO bound form was generated by gently bubbling the sample with CO for 15 seconds, and a spectrum was recorded.

The spectrum for circularly permuted hemoglobin (CpHb) and octomeric circularly permuted hemoglobin (OHb) were nearly identical to the spectrum of native hemoglobin. The absorbance maxima for each ligation state are listed below in nM.

	ABSORBANCE MAXIMA							
	DEOXY Hb		—OXY Hb			—CO Hb		
Native Hb	430	555	414	541	576	419	539	568
CPHb	429	555	414	540	577	419	538	568
OHb	429	554	414	541	576	419	539	568

SIS-PAGE

Polyacrylamide gel electrophoresis was performed on the purified protein samples. Each protein sample was boiled for 5 minutes in 10 mM tris-CHl, pH 8.0, 1 mM EDTA, 2.5% SDS, 5% b-mercaptoethanol, and 0.001% bromophenol-blue. 2 μL of the samples were loaded onto a 10–15% preformed gradient gel (Pharmacia). The gels were run using the Pharmacia PhastSystem for a total of 60 Volt hours at 250 Volts, 10.0 mA, 3.0 Watts, and 15° C. The gels were developed in the PhastSystem developing chamber using the fast coomassie staining technique.

Oxygen Equilibrium Measurements

The oxygen equilibrium measurements taken in a home-made hemeox analyzer (Ron Hernan, Ph.D. Thesis, University of Illinois at Urbana Champaign, 1994) are shown in FIG. **17**. The equilibrium binding curves of native hemoglobin, circularly permuted hemoglobin (CpHb), and octomeric circularly permuted hemoglobin (OHb) in 0.05 M Tris-HCl buffer at pH 7.4 with 0.1M [Cl^-] were performed at 60 μM, 5 μM, and 5 μM in [heme] respectively. P_{50} values and n_{max} values were then calculated for each protein. A bohr effect was determined by measure p_{50} values for each protein at pH 6.5 and 8.5 using 0.05 M Bis Tris with 0.1M [Cl^-] and 0.05M Tris-CHl with 0.1M [Cl^-] respectively. Finally each protein's response to allosteric effectors was measured with the addition of 0.1 mM IHP. Comparisons between the proteins are listed below and indicate that the circularly permuted hemoglobins cooperatively bind oxygen (n_{max}=2) and that they still respond to allosteric effectors (protons and IHP) in a similar manner to native hemoglobin.

	HbA_o	CpHb	Octamer Hb
P_{50} (mmHg)	5.0 ± 0.2	0.9 ± 0.1	0.8 ± 0.1
N_{max}	3.0	2.0	1.9
$\Delta logp_{50}$ ± 0.1 mM IHP	0.9	0.7	0.7
Bohr Effect	−0.5	−0.3	−0.4

Plasma Lifetime Measurements

Intravascular lifetime of the circularly permuted hemoglobin was measured and compared with the lifetime of cell free HbA. HbA was obtained from purification of freshly drawn blood as previously described (Ron Hernan, Ph.D. Thesis, University of Illinois at Urbana Champaign, 1994). Young adult Spaugue Dawley rats, weighing between 150 and 200 grams were used for the experiments. The protein samples were exchanged into a buffer solution consisting of 150 mM NaCl, 5 mM KCl, 2 mM $NaPO_4$, at pH 7.4 using a Sephadex G-25 column. The samples were then concentrated to approximately 1.5 mM in heme. 1 mL of this solution was filter sterilized and injected into each rat through a surgically placed jugular catheter. 200 μL blood samples were withdrawn from the same catheter at 5 minutes as a baseline and at successive time intervals. Plasma was separated from the erythrocytes by centrifugation, and the hemoglobin content was determined with the Plasma Hemoglobin Diagnostic Kit from Sigma. The measurements were then normalized to the 5 minute value and three experiments in three different rats were averaged for each protein. Exponential fits of the data are shown in FIG. **18**, and the $T_{1/2}$ were calculated to be 30 and 90 minutes for HbA and circularly permuted hemoglobin respectively.

APPENDIX A

VARIANTS OF THE ALPHA CHAIN

Residue	Substitution	Hb Name	Major Abnormal Property
1 (NA1)	Val		
2 (NA2)	Leu→Arg	Chongqing	↑ O_2 affinity; unstable
3 (A1)	Ser		
4 (A1)	Pro		
5 (A3)	Ala→Asp	J-Toronto	
	Ala→Pro	Karachi	
6 (A4)	Asp→Ala	Sawara	↑ O_2 affinity
	Asp→Asn	Dunn	↑ O_2 affinity
	Asp→Val	Ferndown	↑ O_2 affinity
	Asp→Tyr	Woodville	↑ O_2 affinity
	Asp→Gly	Swan River	
7 (A5)	Lys→Asn	Tatras	
	Lys→Glu	Kurosaki	
8 (A6)	Thr		
9 (A7)	Asn		
10 (A8)	Val		
11 (A9)	Lys→Glu	Anantharaj	
	Lys→Gln	J-Wenchang-Wuming	
	Lys→Asn	Albany-Suma	
12 (A10)	Ala→Asp	J-Paris-I; J-Aljezur	
13 (A11)	Ala		
14 (A12)	Trp→Arg	Evanston	↑ O_2 affinity
15 (A13)	Gly→Asp	I-Interlaken; J-Oxford; N-Cosenza	
	Gly→Arg	Ottawa; Siam	
16 (A14)	Lys→Glu	I; I-Philadelphia; I-Texas; I-Burlington; I-Skamania	
	Lys→Asn	Beijing	
	Lys→Met	Harbin	↑ O_2 affinity; slightly unstable
17 (A15)	Val		
18 (A16)	Gly→Arg	Handsworth	
	Gly→Asp	Al-Ain Abu Dhabi	
19 (AB1)	Ala→Asp	j-Kurosh	
	Ala→Glu	Tashikuergan	
20 (B1)	His→Tyr	Necker Enfants-Malades	
	His→Gln	Le Lamentin	
	His→Arg	Hobart	
21 (B2)	Ala→Asp	J-Nyanza	
	Ala→Pro	Fontainebleau	
22 (B3)	Gly→Asp	J-Medellin	
22 (B4)	Gly→Gln	Memphis	
	Glu→Lys	Chad	
	Glu→Val	G-Audhali	
	Gly→Gly	Reims	slightly unstable
	Glu→Asp	Lisbon	
24 (B5)	Tyr→His	Luxembourg	unstable
	Tyr→Cys	Ramona	
25 (B6)	Gly		
26 (B7)	Ala→Glu	Shenyang	unstable
27 (B8)	Glu→Gly	Fort Worth	
	Glu→Val	Spanish Town	
	Glu→Lys	Shuangfeng	unstable
	Glu→Asp	Hekinan	
28 (B9)	Ala		
29 (B10)	Leu→Pro	Agrinio	unstable; thalessemic
30 (B11)	Glu→Lys	O-Padova	
	Glu→Gln	G-Honolulu; G-Singapore; G-Chinese; G-Hong Kong	
31 (B12)	Arg→Ser	Prato	unstable
32 (B13)	Met		
33 (B14)	Phe		
34 (B15)	Leu→Arg	Queens; Ogi	
35 (B16)	Ser		
36 (C1)	Phe		
37 (C2)	Pro→Arg	Bourmedès	
38 (C3)	Thr		
39 (C4)	Thr		
40 (C5)	Lys→Glu	Kariya	↑ O_2 affinity; unstable
	Lys→Met	Kanagawa	↑ O_2 affinity
41 (C6)	Thr→Ser	Miyano	↑ O_2 affinity
42 (C7)	Tyr		
43 (CE1)	Phe→Val	Torino	unstable; ↑ O_2 affinity
	Phe→Leu	Hirosaki	unstable
44 (CE2)	Pro→Leu	Milledgeville	↑ O_2 affinity
	Pro→Arg	Kawachi	↑ O_2 affinity
45 (CE3)	His→Arg	Fort de France	↑ O_2 affinity
	His→Gln	Bari	
	His→Asp	Poitiers	↑ O_2 affinity
46 (CE4)	Phe		
47 (CE5)	Asp→Gly	Kokura; Umi	unstable; ↑ O_2 affinity
		Michigan-I and -II; Yukuhashi-II; L-Gaslini; Tagawa-II; Beilinson; Mugino	
	Asp→His	Hasharon; Sinai; Sealy;	unstable

-continued

VARIANTS OF THE ALPHA CHAIN

Residue	Substitution	Hb Name	Major Abnormal Property
		L-Ferrara	
	Asp→Asn	Arya	slightly unstable
	Asp→Ala	Cordele	unstable
	Asp→Tyr	Kurdistan	thalassemic
48 (CE6)	Leu→Arg	Montgomery	
49 (CE7)	Ser→Arg	Savaria	
50 (CE8)	His→Asp	J-Sardegna	
	His→Arg	Aichia	slightly unstable
51 (CE9)	Gly→Asp	J-Abidjan	
	Gly→Arg	Russ	
52 (EI)	Ser		
53 (E2)	Ala→Asp	J-Rovigo	unstable
54 (E3)	Gln→Arg	Shimonoseki; Hikoshima	
	Gln→Glu	Mexico; J; J-Paris-II; Uppsala	
55 (E4)	Val		
56 (E5)	Lys→Thr	Thailand	
	Lys→Glu	Shaare Zedek	
	Lys→Asn	Belliard	
	Lys→Arg	Port Huron	
57 (E6)	Gly→Arg	L-Persian Gulf	
	Gly→Asp	J-Norfold; Kagoshima; Nishik-I; II; III	
58 (E7)	His→Tyr	M-Boston; M-Osaka; Gothenburg; M-Kiskunhalas	↑ O_2 affinity
59 (E8)	Gly→Val	Tottori	unstable
	GIy→Asp	Adana	unstable
60 (E9)	Lys→Asn	Zambia	
	Lys→Glu	Dagestan	
61 (E10)	Lys→Asn	J-Buda	
	Lys→Thr	J-Anatolia	
62 (E11)	Val→Met	Evans	unstable
63 (E12)	Ala→Asp	Pontoise; J-Pontoise	unstable
64 (E13)	Asp→Asn	G-Waimanalo; Aida	
	Asp→His	Q-India	
	Asp→Tyr	Perspolis	
	Asp→Gly	Guangzhou-Hangzhou	
64 (E14)	Ala		
66 (E15)	Leu		
67 (E16)	Thr		
68 (E17)	Asn→Asp	Ube-2	
	Asn→Lys	G-Philadelphia; G-Knoxville-I; StanleyvilIe-I; D-Washington; D-St. Louis; G-Bristol; G-Azakuoli; D-Baltimore	
69 (E18)	Ala		
70 (E19)	Val		
71 (E20)	Ala→Glu	J-Habana	
	Ala→Val	Ozieri	
72 (EF1)	His→Arg	Daneshgah-Tehran	
73 (EF2)	Val		
101 (G8)	Leu		
102 (G9)	Ser→Arg	Manitoba	slightly unstable
103 (G10)	His→Arg	Contaldo	unstable
104 (G11)	Cys→Tyr	Sallanches	
105 (G12)	Leu		
106 (G13)	Leu		
107 (G14)	Val		
108 (G15)	Thr		
109 (G16)	Leu→Arg	Suan-Dok	unstable
110 (G17)	Ala→Asp	Petah Tikva	unstable
	Ala→Thr	Tonosho	slightly unstable; ↑ dissociation;

-continued

VARIANTS OF THE ALPHA CHAIN

Residue	Substitution	Hb Name	Major Abnormal Property
			↑ O_2 affinity
111 (G18)	Ala→Val	Anamosa	
112 (G19)	His→	Hopkins-II	unstable; ↑ O_2 affinity
	His→Arg	Strumica; Serbia	
113 (GH1)	Leu→His	Twin Peaks	
114 (GH2)	Pro→Arg	Chiapas	
	Pro→Leu	Nouakchott	↑ hydrophobicity
	Pro→Ser	Melusine	
115 (GH3)	Ala→Asp	J-Tongariki	
116 (GH4)	Glu→Lys	O-Indonesia; Buginese-X; Oliviere	
	Glu→Ala	Ube-4	
	Glu→Gln	Oleander	
117 (GH5)	Phe		
118 (H1)	Thr		
119 (H2)	Pro		
120 (H3)	Ala→Glu	J-Meerut; J-Birmingham	
121 (H4)	Val→Met	Owari	
122 (H5)	His→Gln	Westmead	
123 (H6)	Ala→Ser	Mulhacen	
124 (H7)	Ser		
125 (H8)	Leu→Pro	Quong Sze	
126 (H9)	Asp→Asn	Tarrant	↑ O_2 affinity
	Asp→His	Sassari	
	Asp→Val	Fukutomi	↑ O_2 affinity
	Asp→Tyr	Montefiore	↑ O_2 affinity
127 (H10)	Lys→Thr	St. Claude	
	Lys→Asn	Jackson	
128 (H11)	Phe		
129 (H12)	Leu→Pro	α-Tunis	unstable; thalessemic
130 (H13)	Ala→Pro	Sun Prairie	unstable
	Ala→Asp	Yuda	↑ O_2 affinity
131 (HI4)	Ser→Pro	Questembert	highly unstable
132 (H15)	Val→Gly	Caen	unstable
133 (H16)	Ser→Arg	Val de Marne; Footscray	sl. ↑ O_2 affinity
134 (H17)	Thr		
135 (H18)	Val→Glu	Pavie	
136 (H19)	Leu→Pro	Bibba	unstable; ↑ dissociation
	Leu→Arg	Toyama	unstable
	Leu→Met	Chicago	
137 (H20)	Thr		
138 (H21)	Ser→Pro	Attleboro	↑ O_2 affinity
139 (HC1)	Lys→Thr	Tokoname	↑ O_2 afflnity
	Lys→Glu	Hanamaki	↑ O_2 affinity
140 (HC2)	Tyr→His	Ethiopia; Rouen	↑ O_2 affinity; ↑ O_2 affinity
141 (HC3)	Arg→Pro	Singapore	
	Arg→His	Suresnes	↑ O_2 affinity
	Arg→Ser	J-Cubujuqui	↑ O_2 affinity
	Arg→Leu	Legnano	↑ O_2 affinity
	Arg→Gly	J-Camagüey	
	Arg→Cys	Nunobiki	↑ O_2 affinity

VARIANTS OF THE BETA CHAIN

Residue	Substitution	Hb Name	Major Abnormal Property
1 (NA1)	Val→Ac-Ala	Raleigh	↓ O_2 affinity; ↓ dissociation
2 (NA2)	His→Arg	Deer Lodge	↑ O_2 affinity
	His→Gln	Okayama	↑ O_2 affinity
	His→Tyr	Fukuoka	

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VARIANTS OF THE BETA CHAIN

Residue	Substitution	Hb Name	Major Abnormal Property
	His→Leu	Graz	
3 (NA3)	Leu		
4 (A1)	Thr		
5 (A2)	Pro→Arg	Warwickshire	
	Pro→Ser	Tyne	
6 (A3)	Glu→Val	S	
	Glu→Lys	C	
	Glu→Ala	G-Makassar	
	Glu→Gln	Machida	
7 (A4)	Glu→Gly	G-San José	mildly unstable
	Glu→Lys	Siriraj	
8 (A5)	Lys→Thr	Rio Grande	
	Lys→Gln	J-Lube	
	Lys→Glu	N-Timone	
9 (A6)	Ser→Cys	Port Alegre	polymerization; ↑ O_2 affinity; ↓ heme-heme
10 (A7)	Ala→Asp	Ankara	
11 (A8)	Val→Ile	Hamilton	
	Val→Asp	Windsor	unstable
	Val→Phe	Washtenaw	↓ O_2 affinity
12 (A9)	Thr		
13 (A10)	Ala→Asp	J-Lens	
14 (A11)	Leu→Arg	Sögn	unstable
	Leu→Pro	Saki	unstable
15 (A12)	Trp→Arg	Belfast	unstable; ↑ O_2 affinity
	Trp→Gly	Randwick	unstable
16 (A13)	Gly→Asp	J-Baltimore; J-Trinidad; J-Ireland; N-New Haven; J-Georgia	
	Gly→Arg	D-Bushman	
17 (A14)	Lys→Glu	Nagasaki	
	Lys→Asn	J-Amiens	
	Lys→Gln	Nikosia	
18 (A15)	Val→Met	Baden	slightly unstable
	Val→Gly	Sinai-Baltimore	Slightly unstable
19 (B1)	Asn→Lys	D-Ouled Rabah	
	Asn→Asp	Alamo	
	Asn→Ser	Malay	
20 (B2)	Val→Met	Olympia	↑ O_2 affinity
	Val→Glu	Trollhattan	↑ O_2 affinity
21 (B3)	Asp→Tyr	Yusa	
	Asp→Gly	Connecticut	↑ O_2 affinity
	Asp→Asn	Cocody	
	Asp→His	Kariskoga	
22 (B4)	Glu→Lys	E-Saskatoon	unstable
	Glu→Gly	G-Taipei	
	Glu→Ala	G-Coushatta; G-Saskatoon; Hsin Chu; G-Taegu	
	Glu→Gln	D-Iran	
	Glu→Val	D-Granada	
23 (B5)	Val→Asp	Strasbourg	↑ O_2 affinity
	Val→Gly	Miyashiro	unstable; ↑ O_2 affinity
	Val→Phe	Palmerston North	↑ O_2 affinity; unstable
24 (B6)	Gly→Arg	Riverdale-Bronx	unstable; ↑ O_2 affinity
	Gly→Val	Savanna	unstable
	Gly→Asp	Moscva	unstable; ↓ O_2 affinity
25 (B7)	Gly→Arg	G-Taiwan Ami	
	Gly→Asp	J-Auckland	Unstable; ↓ O_2 affinity
26 (B8)	Glu→Lys	E	
	Glu→Val	Henri Mondor	slightly unstable
27 (B9)	Ala→Asp	Volga; Drenthe	unstable
	Ala→Ser	Knossos	
	Ala→Val	Grange-Blanche	↑ O_2 affinity
28 (B10)	Leu→Gln	St. Louis	unstable; ferri-Hb; ↑ O_2 affinity
	Leu→Pro	Genova; Hyogo	unstable; ↑ O_2 affinity
	Leu→Arg	Chesterfield	unstable; thalassemic
29 (B11)	Gly→Asp	Lufkin	unstable
30 (B12)	Arg→Ser	Tacoma	unstable; ↓ Bohr and heme-heme
	Arg→Thr	Monroe; Kairouan	
31 (B13)	Leu→Pro	Yokohama	unstable
	Leu→Arg	Hakkari	severely unstable
32 (B14)	Leu→Pro	Perth; Abraham Lincoln; Kobe	unstable
	Leu→Arg	Castilla	unstable
	Leu→Val	Muscat	slightly unstable
	Leu→Gln	Medicine Lake	
33 (B15)	Val		
34 (B16)	Val→Phe	Pitie-Salpetriere	↑ O_2 affinity
35 (C1)	Tyr→Phe	Philly	unstable; ↑ O_2 affinity
36 (C2)	Pro→Thr	Linköping; Meilahti; Finiandia	unstable; ↑ O_2 affinity; ↑ O_2 affinity
	Pro→Ser	North Chicago	↑ O_2 affinity
	Pro→Arg	Sunnybrook	
37 (C3)	Trp→Ser	Hirose	↑ O_2 affinity; ↑ dissociation
	Trp→Arg	Rothschiid	↓ O_2 affinity
	Trp→Gly	Howick	↑ O_2 affinity
38 (C4)	Thr→Pro	Hazebrouck	unstable; ↓ O_2 affinity
39 (C5)	Gln→Lys	Alabama	
	Gln→Glu	Vaasa	unstable
	Gln→Arg	Tianshui	
40 (C6)	Arg→Lys	Athens-GA; Waco	↑ O_2 affinity
	Arg→Ser	Austin	↑ O_2 affinity; ↑ dissociation
41 (C7)	Phe→Tyr	Mequon	
	Phe→Ser	Denver	↓ O_2 affinity; cyanotic
42 (CD1)	Phe→Ser	Hammersmith; Chiba	unstable; ↓ O_2 affinity
	Phe→Leu	Louisville; Bucuresti	unstable; ↓ O_2 affinity
	Phe→Val	Sendagi; Warsaw	unstable; ↓ O_2 affinity
43 (CD2)	Glu→Ala	G-Galveston; G-Port Arthur; G-Texas	
	Glu→Gln	Hoshida; Chaya	
44 (CD3)	Ser→Cys	Mississippi	
45 (CD4)	Phe→Ser	Cheverly	unstable; ↓ O_2 affinity; ↓ Bohr effect
	Phe→Cys	Arta	unstable; ↓ O_2 affinity; thalassemic
46 (CD5)	Gly→Glu	K-Ibadan	
	Gly→Arg	Gainesville-GA	
47 (CD6)	Asp→Asn	G-Copenhagen	
	Asp→Gly	Gavello	
	Asp→Ala	Avicenna	
	Asp→Tyr	Maputo	
48 (CD7)	Leu→Arg	Okaloosa	unstable; ↓ O_2 affinity

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VARIANTS OF THE BETA CHAIN

Residue	Substitution	Hb Name	Major Abnormal Property
	Leu→Pro	Bab-Saadoun	slightly unstable
49 (CD8)	Ser→Phe	Las Palmas	slightly unstable
50 (D1)	Thr→Lys	Edmonton	
51 (D2)	Pro→Arg	Willamette	↑ O_2 affinity; unstable
52 (D3)	Asp→Asn	Osu Christiansborg	
	Asp→Ala	Ocho Rios	
	Asp→His	Summer Hill	
53 (D4)	Ala		
54 (D5)	Val→Asp	Jacksonville	Unstable; ↑ O_2 affinity
55 (D6)	Met→Lys	Matera	unstable
56 (D7)	Gly→Asp	J-Bangkok; J-Meinung; J-Korat; J-Manado	
	Gly→Arg	Hamadan	
57 (E1)	Asn→Lys	G-Ferrara	unstable
	Asn→Asp	J-Dalos	
58 (E2)	Pro→Arg	Dhofar; Yukuhashi	
59 (E3)	Lys→Glu	I = High Wycombe	
	Lys→Thr	J-Kaohsiung; J-Honolulu	
	Lys→Asn	J-Lome	↑ autooxidation
60 (E4)	Val→Leu	Yatsushiro	
	Val→Ala	Collingwood	unstable
	Val→glu	Cagliari	unstable; thalassemic
61 (E5)	Lys→Glu	N-Seattle	
	Lys→Asn	Hikari	
	Lys→Met	Bologna	↓ O_2 affinity
62 (E6)	Ala→Pro	Duarte	unstable; ↑ O_2 affinity
63 (E7)	His→Arg	Zürich	unstable; ↑ O_2 affinity
	His→Tyr	M-Saskatoon; M-Emory; M-Kurume; M-Hida; M-Radom; M-Arhus; M-Chicago; Leipzig; Horlein-Weber; Novi Sad; M-Erlangen	ferri-Hb; ↑ O_2 affinity
	His→Pro	Bicêtre	unstable; ↑ autooxidizing
64 (E8)	Gly→Asp	J-Calabria; J-Bari; J-Cosenza	unstable; ↑ O_2 affinity
65 (E9)	Lys→Asn	J-Sicilia	
	Lys→Gln	J-Cairo	↓ O_2 affinity; ↑ autooxidation
	Lys→Met	J-Antakya	
66 (E10)	Lys→Glu	I-Toulouse	unstable; Ferri-Hb
	Lys→Thr	Chico	↓ O_2 affinity
67 (E11)	Val→Asp	Bristol	unstable; ↓ O_2 affinity
	Val→Glu	M-Milwaykee-I	ferri-Hb; ↓ O_2 affinity
	Val→Ala	Sydney	unstable
	Val→Met	Alesha	unstable
	Val→Gly	Manukau	unstable; hemolytic anemia; thalassemic
68 (E12)	Leu→Pro	Mizuho	unstable
	Leu→His	Brisbane; Great Lakes	↑ O_2 affinity; (?) unstable
69 (E13)	Gly→Asp	J-Cambridge; J-Rambam	
	Gly→Ser	City of Hope	
	Gly→Arg	Kenitra	
70 (E14)	Ala→Asp	Seattle	↓ O_2 affinity; unstable
71 (E15)	Phe→Ser	Christchurch	unstable
72 (E16)	Ser→Arg	Headington	↑ O_2 affinity; thalassemic
73 (E17)	Asp→Tyr	Vancouver	↓ O_2 affinity
	Asp→Asn	Korle-Bu; G-Accra	↓ O_2 affinity
	Asp→Val	Mobile	↓ O_2 affinity
	Asp→Gly	Tilburg	↓ O_2 affinity
74 (E18)	Gly→Val	Bushwick	unstable
	Gly→Asp	Shepherds Bush	unstable; ↑ O_2 affinity
	Gly→Arg	Aalborg	unstable
75 (E19)	Leu→Pro	Atlanta	unstable
	Leu→Arg	Pasadena	unstable; ↑ O_2 affinity
76 (E20)	Ala→Asp	J-Chicago	
	Ala→Pro	Calais	↓ O_2 affinity; ↑ met-Hb formation
77 (EF1)	His→Asp	J-Iran	
	His→Tyr	Fukuyama	
78 (EF2)	Leu→Arg	Quin-Hai	
79 (EF3)	Asp→Gly	G-His-Tsou	↑ O_2 affinity
	Asp→Tyr	Tampa	
	Asp→His	Tigraye	↑ O_2 affinity
	Asp→Asn	Yaizu	
80 (EF4)	Asn→Lys	G-Szuhu; Gifu	
81 (EF5)	Leu→Arg	Baylor	unstable; ↑ O_2 affinity
	Leu→His	La Roche-Sur-Yon	unstable; ↑ O_2 affinity
	(β Asn partially deaminated)		
82 (EF6)	Lys→Asn→Asp	Providence	↓ O_2 affinity
	Lys→Thr	Rahere	↑ O_2 affinity
	Lys→Met	Helsinki	↑ O_2 affinity
83 (EF7)	Gly→Cys	Ta-Li	slightly unstable; polymerization
	Gly→Asp	Pyrgos; Misunami	slightly ↓ O_2 affinity
	Gly→Arg	Muskegon	
84 (EF8)	Thr→Ile	Kofu	
85 (F1)	Phe→Ser	Buenos Aires; Bryn Mawr	unstable; ↑ O_2 affinity
86 (F2)	Ala→Asp	Olomouc	↑ O_2 affinity
87 (F3)	Thr→Lys	D-Ibadan	
	Thr→Ile	Quebec-Chori	
	Thr→Pro	Valletta	
88 (F4)	Leu→Arg	Borks	unstable
	Leu→Pro	Santa Ana	unstable
89 (F5)	Ser→Asn	Creteil	↑ O_2 affinity
	Ser→Arg	Vanderbilt	↑ O_2 affinity
	Ser→Thr	Villaverde	↑ O_2 affinity
90 (F6)	Glu→Lys	Agenogi	↓ O_2 affinity
	Glu→Gly	Roseau-Point à Pitre	↓ O_2 affinity; unstable
	Glu→Asp	Pierre-Bénite	↑ O_2 affinity
91 (F7)	Leu→Pro	Sabine	unstable
	Leu→Arg	Caribbean	unstable; ↓ O_2 affinity
92 (F8)	His→Tyr	M-Hyde Park; M-Akita	ferri-Hb
	His→Gln	St. Etienne; Istanbul	unstable; ↑ O_2 affinity ↑ dissociation
	His→Asp	J-Altgeld Gardens	unstable
	His→Pro	Newcastle	unstable

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VARIANTS OF THE BETA CHAIN

Residue	Substitution	Hb Name	Major Abnormal Property
	His→Arg	Mozhaisk	unstable; ↑ O_2 affinity
	His→Asn→Asp	Redondo; Isehara	unstable
93 (F9)	Cys→Arg	Okazaki	↑ O_2 affinity; unstable
94 (FG1)	Asp→His	Barcelona	↑ O_2 affinity
	Asp→Asn	Bunbury	↑ O_2 affinity
	Asp→Gly	Chandigarh	
95 (FG2)	Lys→Glu	N-Baltimore; Hopkins-I; Jenkins; N-Memphis; Kenwood	
	Lys→Met	J-Cordoba	
	Lys→Asn	Detroit	
96 (FG3)	Leu→Val	Regina	↑ O_2 affinity
97 (FG4)	His→Gln	Malmö	↑ O_2 affinity
	His→Leu	Wood	↑ O_2 affinity
	His→Pro	Nagoya	unstable; ↑ O_2 affinity
	His→Tyr	Moriguchi	
98 (FG5)	Val→Met	Köln; San Francisco (Pacific); Ube-I	unstable; ↑ O_2 affinity
	Val→Gly	Nottingham	unstable; ↑ O_2 affinity
	Val→Ala	Djelfa	unstable; ↑ O_2 affinity
	Val→Glu	Mainz	unstable
99 (G1)	Asp→Asn	Kempsey	↑ O_2 affinity
	Asp→His	Yakima	↑ O_2 affinity
	Asp→Ala	Radcliffe	↑ O_2 affinity
	Asp→Tyr	Ypsilanti	↑ O_2 affinity
	Asp→Gly	Hotel-Dieu	↑ O_2 affinity
	Asp→Val	Chemilly	↑ O_2 affinity
	Asp→Glu	Coimbra; Ingelheim	↑ O_2 affinity; polycythemia
100 (G2)	Pr→Leu	Brigham	↑ O_2 affinity
	Pro→Arg	New Mexico	
101 (G3)	Glu→Lys	British Columbia	↑ O_2 affinity
	Glu→Gln	Rush	unstable
	Glu→Gly	Alberta	↑ O_2 affinity
	Gl→Asp	Potomac	↑ O_2 affinity
102 (G4)	Asn→Lys	Richmond	asymmetric hybrids
	Asn→Thr	Kansas	↓ O_2 affinity; ↑ dissociation
	Asn→Ser	Beth Israel	unstable; ↓ O_2 affinity
	Asn→Tyr	Saint Mandé	↓ O_2 affinity
103 (G5)	Phe→Leu	Heathrow	↑ O_2 affinity
	Phe→Ile	Saint Nazaire	↑ O_2 affinity
104 (G6)	Arg→Ser	Camperdown	slightly unstable
	Arg→Thr	Sherwood Forest	
105 G7)	Leu→Phe	South Milwaukee	↑ O_2 affinity
106 (G8)	Leu→Pro	Southampton; Casper	unstable; ↑ O_2 affinity
	Leu→Gln	Tübingen	unstable; ↑ O_2 affinity
	Leu→Arg	Terre Haute	very unstable; formerly incorrectly identified as Hb Indianapolis [β112(G14) Cys→Arg]; see ref. 318
107 (G9)	Gly→Arg	Burke	unstable; ↓ O_2 affinity
108 (G10)	Asn→Asp	Yoshizuka	↓ O_2 affinity

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VARIANTS OF THE BETA CHAIN

Residue	Substitution	Hb Name	Major Abnormal Property
	Asn→Lys	Presbyterian	↓ O_2 affinity; unstable
109 (G11)	Val→Met	San Diego	↑ O_2 affinity
	Val→Leu	Johnstown	↑ O_2 affinity
110 (G12)	Lsu→Pro	Showa-Yakushiji	
111 (G13)	Val→Phe	Peterborough	unstable; ↓ O_2 affinity
	Val→Ala	Stanmore	unstable; ↓ O_2 affinity
112 (G14)	Cys→Arg	Indianapolis	(See also β106-Terre Haute)
	Cys→Tyr	Yahata	
113 (G15)	Val→Glu	New York; Kaohslung	unstable; ↓ O_2 affinity
114 (G16)	Leu→Met	Zengcheng	
	Leu→Pro	Durham-N.C.	unstable; thalessemic
115 (G17)	Ala→Pro	Madrid	unstable
	Ala→Asp	Hradec Kralove (HK)	highly unstable; thalessemic
116 (G18)	His→Gln	Hafnia	
117 (G19)	His→Arg	P-Glaveston	
	His→Pro	Saitama	unstable
118 (GH1)	Phe→Tyr	Minneapolis-Laos	
119 (GH2)	Gly→Asp	Fannin-Lubbock	slightly unstable
	Gly→Val	Bougardirey-Mali	slightly unstable
	Gly→Ala	Iowa	
120 (GH3)	Lys→Glu	Hijiyama	
	Lys→Asn	Riyadh; Karatsu	
	Lys→Gln	Takamatsu	
	Lys→Ile	Jianghua	
121 (GH4)	Glu→Gln	D-Los Angeles; D-Punjab; D-North Carolina; D-Portugal; Oak Ridge; D-Chicago	↓ O_2 affinity
	Glu→Lys	O-Arab; Egypt	
	Glu→Val	Beograd; D-Camperdown	
	Glu→Gly	St. Francis	
	Glu→Ala	D-Neath	
122 (GH5)	Phe		
123 (H1)	Thr→Ile	Villejuif	
124 (H2)	Pro→Arg	Khartoum	unstable
	Pro→Gln	Ty Gard	↑ O_2 affinity
	Pro→Ser	β-Tunis	
125 (H3)	Pro		
126 (H4)	Val→Glu	Hofu	unstable
	Val→Ala	Beirut	stable
	Val→Gly	Dhonburi; Neapolis	unstable; thalessemic unstable
127 (H5)	Gln→Glu	Complutense	slightly unstable
	Gly→Lys	Brest	unstable
	Gln→Arg	Dieppe	unstable; anemic
128 (H6)	Ala→Asp	J-Guantanamo	unstable
129 (H7)	Ala→Asp	J-Taichung	
	Ala→Glu or Asp	K-Cameroon	
129 (H7)	Ala→Pro	Crete	unstable; ↑ O_2 affinity
	Ala→Val	La Desirade	unstable; ↓ O_2 affinity
130 (H8)	Tyr→Asp	Wien	unstable
	Tyr→Ser	Nevers	
131 (H9)	Gln→Gln	Camden; Tokuchi; Motown	
	Gln→Lys	Shelby; formerly: Leslie; Deaconess	unstable
	Gln→Pro	Shanghai	unstable

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VARIANTS OF THE BETA CHAIN

Residue	Substitution	Hb Name	Major Abnormal Property
	Gln→Arg	Sarrebourg	unstable
132 (H10)	Lys→gln	K-Woolwich	
	Lys→Asn	Yamagata	slightly ↓ O_2 affinity
133 (H11)	Val→Leu	Extremadura	
134 (H12)	Val→Glu	North Shore; North Shore-Caracas	unstable
135 (H13)	Ala→Pro	Altdorf	unstable; ↑ O_2 affinity
	Ala→Glu	Beckman	unstable; ↓ O_2 affinity
136 (H14)	Gly→Asp	Hope	unstable; ↓ O_2 affinity
137(H15)	Val		
138 (H16)	Ala→Pro	Brockton	unstable
139 (H17)	Asn→Asp	Geelong	unstable
	Asn→Lys	Hinsdale	
	Asn→Tyr	Aurora	↑ O_2 affinity
140 (H18)	Ala→Thr	Saint Jacques	↑ O_2 affinity
	Ala→Asp	Himeji	unstable; ↓ O_2 affinity
	Ala→Val	Puttelange	↑ O_2 affinity
141 (H19)	Leu→Arg	Olmsted	Unstable
142 (H20)	Ala→Asp	Ohio	↑ O_2 affinity; reduced Bohr effect
	Ala→Pro	Toyoake	unstable; ↑ O_2 affinity
143 (H21)	His→Arg	Abruzzo	↑ O_2 affinity
	His→Gln	Little Rock	↑ O_2 affinity
	Hi→Pro	Syracuse	↑ O_2 affinity
	His→Asp	Rancho Mirage	
144 (HC1)	Lys→Asn	Andrew-Minneapolis	↑ O_2 affinity
	Lys→Glu	Mito	↑ O_2 affinity
145 (HC2)	Try→His	Bethesda	↑ O_2 affinity
	Tyr→Cys	Rainier	↑ O_2 affinity; alkali resistant
	Tyr→Asp	Fort Gordon; Osler; Nancy	↑ O_2 affinity
	Tyr→Term	McKees Rocks	↑↑ O_2 affinity
146 (HC3)	His→Asp	Hiroshima	↑ O_2 affinity
	His→Pro	York	↑ O_2 affinity
	His→Arg	Cochin-Port Royal	
	His→Leu	Cowtown	↑ O_2 affinity
	His→Gln	Kodaira	↑ O_2 affinity

VARIANTS OF THE GAMMA CHAIN

Residue	Substitution	Hb Name	Major Abnormal Property
Variants of the $^{G}\gamma$Chain			
1 (NA1)	Gly→Cys	-Malaysia	
5 (A2)	Glu→Gly	F-Meinohama	
7 (A4)	Asp→Asn	F-Auckland	
8 (A5)	Lys→Glu or Gln	F-Albaicin	
12 (A9)	Thr→Arg	F-Heather	
15 (A12)	Trp→Arg	F-Catalonia	
16 (A13)	Gly→Arg	F-Melbourne	
21 (B3)	Glu→Gln	F-Fuchu	
	Glu→Lys	F-Saskatoon	
22 (B4)	Asp→Gly	F-Urumqi	
	Asp→Val	F-Granada	
25 (B7)	Gly→Glu	F-Cosenza	
26 (B8)	Glu→Lys	F-Oakland	
34 (B16)	Val→Ile	F-Tokyo	

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VARIANTS OF THE GAMMA CHAIN

Residue	Substitution	Hb Name	Major Abnormal Property
40 (C6)	Arg→Lys	F-Austell	
44 (CD3)	Ser→Arg	F-Lodz	
55 (D6)	Met→Arg	F-Kingston	
59 (E3)	Lys→Gln	F-Sacromonte; F-Foch	
	Lys→Glu	F-Emirates	
63 (E7)	His→Tyr	F-M-Osaka	metHb
65 (E9)	Lys→Asn	F-Clarke	
66 (E10)	Lys→Arg	F-Shanghai	
	Lys→Gln	F-Brooklyn	
72 (E16)	Gly→Arg	F-Minco	
75 (E19)	Ile→Thr	F-Sassari	
77 (EF1)	His→Arg	F-Kennestone	
80 (EF4)	Asp→Asn	F-Marietta	
92 (F8)	His→Tyr	F-M-Fort Ripley	cyanosis
94 (FG1)	Asp→Asn	F-Columbus-GA	
101 (G3)	Glu→Lys	F-La Grange	
104 (G6)	Lys→Asn	F-Macedonia-II	
117 (G19)	His→Arg	F-Malta-I	
120 (GH3)	Lys→Gln	F-Caltech	
121 (GH4)	Glu→Lys	F-Carlton	
125 (H3)	Glu→Ala	F-Port Royal	
130 (H8)	Trp→Gly	F-Poole	unstable
146 (HC3)	His→Tyr	F-Onoda	↑ O_2 affinity
Variants of the $^{A}\gamma$Chain			
2 (NA2)	His→Gln	F-Macedonia-I	
5 (A2)	Glu→Lys	F-Texas-I	
6 (A3)	Glu→Gly	F-Kotobuku; F-Izumi	
	Glu→Gln	F-Pordenone	
12 (A9)	Thr→Arg	F-Calluna	
22 (B4)	Asp→Gly	F-Kuala Lumpur	
36 (C2)	Pr→Arg	F-Pendergrass	
37 (C3)	Trp→gly	F-Cobb	
39 (C5)	Gln→Arg	F-Bonaire-GA	
40 (C6)	Arg→Lys	F-Woodstock	
53 (D4)	Ala→Asp	F-Beech Island	
61 (E5)	Lys→Glu	F-Jamaica	
72 (E16)	Gly→Arg	F-Iwata	
73 (E17)	Asp→His	F-Xin-Su	
75 (E19)	Ile→Thr	F-Sardinia (AγT)	
79 (EF3)	Asp→Asn	F-Dammam	
80 (EF4)	Asp→Tyr	F-Victoria Jubilee	
97 (FG4)	His→Arg	F-Dickinson	
121 (GH4)	Glu→Lys	F-Hull	
128 (H6)	Ala→Thr	F-Baskent	
134 (H12)	Val→Met	F-Jiangsu	
Variants of the $^{A}\gamma^{T}$ Chain			
25 (B7)	Gly→Arg	F-Xinjiang	unstable
43 (CD2)	Asp→Asn	F-Fukuyama	
73 (E17)	Asp→Asn	F-Forest Park	
80 (EF4)	Asp→Asn	F-Yamaguchi	
121 (GH4)	Glu→Lys	F-Siena	
136 (H14)	Ala→Gly	F-Charlotte	
Others			
6 (A3)	Glu→Lys	F-Texas-II	
12 (A9)	Thr→Lys	F-Alexandra	
108 (G10)	Asn→Lys	F-Ube	

VARIANTS OF THE DELTA CHAIN

Residue	Subsitution	Hb Name	Major Abnormal Property
1 (NA1)	Val→Ala	A_2-Niigata	
2 (NA2)	His→Arg	A_2-Sphakia	

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VARIANTS OF THE DELTA CHAIN

Residue	Subsitution	Hb Name	Major Abnormal Property
12 (A9)	Asn→Lys	A_2-NYU	
16 (A13)	Gly→Arg	$A_2^1(B_2)$	
20 (B2)	Val→Glu	A_2-Roosevelt	
22 (B4)	Ala→Glu	A_2-Flatbush	
24 (B6)	Gly→Asp	A_2-Victoria	
25 (B7)	Gly→Asp	A_2-Yokoshima	
26 (B8)	Glu→Asp	A_2-Puglia	
27 (B9)	Ala→Ser	A_2-Yialousa	
43 (CD2)	Glu→Lys	A_2-Melbourne	
47 (CD6)	Asp→Val	A_2-Parkville	
51 (D2)	Pro→Arg	A_2-Adria	
69 (E13)	Gly→Arg	A_2-Indonesia	
75 (E19)	Leu→Val	A_2-Grovetown	

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VARIANTS OF THE DELTA CHAIN

Residue	Subsitution	Hb Name	Major Abnormal Property
90 (F6)	Glu→Val	A_2-Honai	
93 (F9)	Cys→Gly	A_2-Sant[1] Antioco	
98 (FG5)	Val→Met	A_2-Wrens	unstable
99 (G1)	Asp→Asn	A_2-Canada	↑ O_2 affinity
116 (G18)	Arg→His	A_2-Coburg	
	Arg→Cys	A_2-Troodos	thalassemic
117 (G19)	Asn→Asp	A_2-Liangcheng	
121 (GH4)	Glu→Val	A_2-Manzanares	unstable
125 (H3)	Gln→Glu	A_2-Zagreb	
136 (H14)	Gly→Asp	A_2-Babinga	
141 (H19)	Leu→Pro	A_2-Pelendri	thalassemic
142 (H20)	Ala→Asp	A_2-Fitzroy	

SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 19

<210> SEQ ID NO 1
<211> LENGTH: 79
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Cassette

<400> SEQUENCE: 1

gacaagactg aagatttatg gcgccacaag acagaggccg tctgttttga ttgcaatttc 60

gacgaacccc atttcaacc 79

<210> SEQ ID NO 2
<211> LENGTH: 87
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Cassette

<400> SEQUENCE: 2

tcgactgttc tgacttctaa ataccgcggt gttctgtctc cggcagacaa aactaacgtt 60

aaagctgctt ggggtaaagt tggagct 87

<210> SEQ ID NO 3
<211> LENGTH: 894
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: CDS
<222> LOCATION: (34)..(882)

<400> SEQUENCE: 3

tctagaataa ctaactaaag gagaacaaca acc atg ctg tct ccg gca gac aaa 54
Met Leu Ser Pro Ala Asp Lys
1 5

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act aac gtt aaa gct gct tgg ggt aaa gtt gga gct cat gct ggt gaa      102
Thr Asn Val Lys Ala Ala Trp Gly Lys Val Gly Ala His Ala Gly Glu
         10                  15                  20

tac ggt gct gaa gca ctc gag cgt atg ttc ctg tct ttc ccg act act      150
Tyr Gly Ala Glu Ala Leu Glu Arg Met Phe Leu Ser Phe Pro Thr Thr
     25                  30                  35

aaa acg tac ttc ccg cat ttc gac ctg tct cat gga tcc gct cag gtt      198
Lys Thr Tyr Phe Pro His Phe Asp Leu Ser His Gly Ser Ala Gln Val
 40                  45                  50                  55

aaa ggt cat ggt aaa aaa gtt gct gac gcg ttg act aac gct gtt gct      246
Lys Gly His Gly Lys Lys Val Ala Asp Ala Leu Thr Asn Ala Val Ala
                 60                  65                  70

cat gtt gac gac atg ccg aac gct ctg tcc gct ctg tca gat ctt cat      294
His Val Asp Asp Met Pro Asn Ala Leu Ser Ala Leu Ser Asp Leu His
             75                  80                  85

gct cat aaa ctg cgc gtt gac ccg gta aac ttc aag ctt ctg tct cat      342
Ala His Lys Leu Arg Val Asp Pro Val Asn Phe Lys Leu Leu Ser His
         90                  95                 100

tgc ctg ctg gtt act ctg gct gct cat ctg ccg gca gaa ttc act ccg      390
Cys Leu Leu Val Thr Leu Ala Ala His Leu Pro Ala Glu Phe Thr Pro
    105                 110                 115

gct gtt cat gct tct ctg gat aaa ttc ctg gct tct gtg tcg act gtt      438
Ala Val His Ala Ser Leu Asp Lys Phe Leu Ala Ser Val Ser Thr Val
120                 125                 130                 135

ctg act tct aaa tac cgc ggt gtt ctg tct ccg gca gac aaa act aac      486
Leu Thr Ser Lys Tyr Arg Gly Val Leu Ser Pro Ala Asp Lys Thr Asn
                140                 145                 150

gtt aaa gct gct tgg ggt aaa gtt gga gct cat gct ggt gaa tac ggt      534
Val Lys Ala Ala Trp Gly Lys Val Gly Ala His Ala Gly Glu Tyr Gly
            155                 160                 165

gct gaa gca ctc gag cgt atg ttc ctg tct ttc ccg act act aaa acg      582
Ala Glu Ala Leu Glu Arg Met Phe Leu Ser Phe Pro Thr Thr Lys Thr
        170                 175                 180

tac ttc ccg cat ttc gac ctg tct cat gga tcc gct cag gtt aaa ggt      630
Tyr Phe Pro His Phe Asp Leu Ser His Gly Ser Ala Gln Val Lys Gly
    185                 190                 195

cat ggt aaa aaa gtt gct gac gcg ttg act aac gct gtt gct cat gtt      678
His Gly Lys Lys Val Ala Asp Ala Leu Thr Asn Ala Val Ala His Val
200                 205                 210                 215

gac gac atg ccg aac gct ctg tcc gct ctg tca gat ctt cat gct cat      726
Asp Asp Met Pro Asn Ala Leu Ser Ala Leu Ser Asp Leu His Ala His
                220                 225                 230

aaa ctg cgc gtt gac ccg gta aac ttc aag ctt ctg tct cat tgc ctg      774
Lys Leu Arg Val Asp Pro Val Asn Phe Lys Leu Leu Ser His Cys Leu
            235                 240                 245

ctg gtt act ctg gct gct cat ctg ccg gca gaa ttc act ccg gct gtt      822
Leu Val Thr Leu Ala Ala His Leu Pro Ala Glu Phe Thr Pro Ala Val
        250                 255                 260

cat gct tct ctg gat aaa ttc ctg gct tct gtg tcg act gtt ctg act      870
His Ala Ser Leu Asp Lys Phe Leu Ala Ser Val Ser Thr Val Leu Thr
    265                 270                 275

tct aaa tac cgt taatgactgc ag                                       894
Ser Lys Tyr Arg
280
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<210> SEQ ID NO 4
<211> LENGTH: 283
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

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<400> SEQUENCE: 4

Met Leu Ser Pro Ala Asp Lys Thr Asn Val Lys Ala Ala Trp Gly Lys
1 5 10 15

Val Gly Ala His Ala Gly Glu Tyr Gly Ala Glu Ala Leu Glu Arg Met
20 25 30

Phe Leu Ser Phe Pro Thr Thr Lys Thr Tyr Phe Pro His Phe Asp Leu
35 40 45

Ser His Gly Ser Ala Gln Val Lys Gly His Gly Lys Lys Val Ala Asp
50 55 60

Ala Leu Thr Asn Ala Val Ala His Val Asp Asp Met Pro Asn Ala Leu
65 70 75 80

Ser Ala Leu Ser Asp Leu His Ala His Lys Leu Arg Val Asp Pro Val
85 90 95

Asn Phe Lys Leu Leu Ser His Cys Leu Leu Val Thr Leu Ala Ala His
100 105 110

Leu Pro Ala Glu Phe Thr Pro Ala Val His Ala Ser Leu Asp Lys Phe
115 120 125

Leu Ala Ser Val Ser Thr Val Leu Thr Ser Lys Tyr Arg Gly Val Leu
130 135 140

Ser Pro Ala Asp Lys Thr Asn Val Lys Ala Ala Trp Gly Lys Val Gly
145 150 155 160

Ala His Ala Gly Glu Tyr Gly Ala Glu Ala Leu Glu Arg Met Phe Leu
165 170 175

Ser Phe Pro Thr Thr Lys Thr Tyr Phe Pro His Phe Asp Leu Ser His
180 185 190

Gly Ser Ala Gln Val Lys Gly His Gly Lys Lys Val Ala Asp Ala Leu
195 200 205

Thr Asn Ala Val Ala His Val Asp Asp Met Pro Asn Ala Leu Ser Ala
210 215 220

Leu Ser Asp Leu His Ala His Lys Leu Arg Val Asp Pro Val Asn Phe
225 230 235 240

Lys Leu Leu Ser His Cys Leu Leu Val Thr Leu Ala Ala His Leu Pro
245 250 255

Ala Glu Phe Thr Pro Ala Val His Ala Ser Leu Asp Lys Phe Leu Ala
260 265 270

Ser Val Ser Thr Val Leu Thr Ser Lys Tyr Arg
275 280

<210> SEQ ID NO 5
<211> LENGTH: 894
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 5

agatcttatt gattgatttc ctcttgttgt tggtacgaca gaggccgtct gttttgattg 60

caatttcgac gaaccccatt tcaacctcga gtacgaccac ttatgccacg acttcgtgag 120

ctcgcataca aggacagaaa gggctgatga ttttgcatga agggcgtaaa gctggacaga 180

gtacctaggc gagtccaatt tccagtacca ttttttcaac gactgcgcaa ctgattgcga 240

caacgagtac aactgctgta cggcttgcga gacaggcgag acagtctaga agtacgagta 300

tttgacgcgc aactgggcca tttgaagttc gaagacagag taacggacga ccaatgagac 360

cgacgagtag acggccgtct taagtgaggc cgacaagtac gaagagacct atttaaggac 420

cgaagacaca gctgacaaga ctgaagattt atggcgccac aagacagagg ccgtctgttt 480

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tgattgcaat ttcgacgaac cccatttcaa cctcgagtac gaccacttat gccacgactt 540

cgtgagctcg catacaagga cagaaagggc tgatgatttt gcatgaaggg cgtaaagctg 600

gacagagtac ctaggcgagt ccaatttcca gtaccatttt ttcaacgact gcgcaactga 660

ttgcgacaac gagtacaact gctgtacggc ttgcgagaca ggcgagacag tctagaagta 720

cgagtatttg acgcgcaact gggccatttg aagttcgaag acagagtaac ggacgaccaa 780

tgagaccgac gagtagacgg ccgtcttaag tgaggccgac aagtacgaag agacctattt 840

aaggaccgaa gacacagctg acaagactga agatttatgg caattactga cgtc 894

<210> SEQ ID NO 6
<211> LENGTH: 82
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Cassette

<400> SEQUENCE: 6

ctagaataac taactaaagg agaacaacaa ccatgtctca tggttccgct caggttaagg 60

gccatggtaa aaaagttgct ga 82

<210> SEQ ID NO 7
<211> LENGTH: 82
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Cassette

<400> SEQUENCE: 7

ttattgattg atttcctctt gttgttggta cagagtacca aggcgagtcc aattcccggt 60

accatttttt caacgactgc gc 82

<210> SEQ ID NO 8
<211> LENGTH: 70
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Cassette

<400> SEQUENCE: 8

tcgagcgcat gttcctgtct ttcccgacta ctaaaacgta cttcccgcat ttcgacctgt 60

aatgactgca 70

<210> SEQ ID NO 9
<211> LENGTH: 61
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Cassette

<400> SEQUENCE: 9

gcgtacaagg acagaaaggg ctgatgattt tgcatgaagg gcgtaaagct ggacattact 60

g 61

<210> SEQ ID NO 10
<211> LENGTH: 900
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: CDS
<222> LOCATION: (34)..(888)

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<400> SEQUENCE: 10

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tctagaataa ctaactaaag gagaacaaca acc atg tct cat ggt tcc gct cag      54
                                   Met Ser His Gly Ser Ala Gln
                                     1               5

gtt aag ggc cat ggt aaa aaa gtt gct gac gcg ttg act aac gct gtt      102
Val Lys Gly His Gly Lys Lys Val Ala Asp Ala Leu Thr Asn Ala Val
         10                  15                  20

gct cat gtt gac gac atg ccg aac gct ctg tcc gct ctg tca gat ctt      150
Ala His Val Asp Asp Met Pro Asn Ala Leu Ser Ala Leu Ser Asp Leu
     25                  30                  35

cat gct cat aaa ctg cgc gtt gac ccg gta aac ttc aag ctt ctg tct      198
His Ala His Lys Leu Arg Val Asp Pro Val Asn Phe Lys Leu Leu Ser
 40                  45                  50                  55

cat tgc ctg ctg gtt act ctg gct gct cat ctg ccg gca gaa ttc act      246
His Cys Leu Leu Val Thr Leu Ala Ala His Leu Pro Ala Glu Phe Thr
                 60                  65                  70

ccg gct gtt cat gct tct ctg gat aaa ttc ctg gct tct gtg tcg act      294
Pro Ala Val His Ala Ser Leu Asp Lys Phe Leu Ala Ser Val Ser Thr
             75                  80                  85

gtt ctg act tct aaa tac cgc ggt gtt ctg tct ccg gca gac aaa act      342
Val Leu Thr Ser Lys Tyr Arg Gly Val Leu Ser Pro Ala Asp Lys Thr
         90                  95                 100

aac gtt aaa gct gct tgg ggt aaa gtt gga gct cat gct ggt gaa tac      390
Asn Val Lys Ala Ala Trp Gly Lys Val Gly Ala His Ala Gly Glu Tyr
    105                 110                 115

ggt gct gaa gca ctc gag cgt atg ttc ctg tct ttc ccg act act aaa      438
Gly Ala Glu Ala Leu Glu Arg Met Phe Leu Ser Phe Pro Thr Thr Lys
120                 125                 130                 135

acg tac ttc ccg cat ttc gac ctg tct cat gga tcc gct cag gtt aaa      486
Thr Tyr Phe Pro His Phe Asp Leu Ser His Gly Ser Ala Gln Val Lys
                140                 145                 150

ggt cat ggt aaa aaa gtt gct gac gcg ttg act aac gct gtt gct cat      534
Gly His Gly Lys Lys Val Ala Asp Ala Leu Thr Asn Ala Val Ala His
            155                 160                 165

gtt gac gac atg ccg aac gct ctg tcc gct ctg tca gat ctt cat gct      582
Val Asp Asp Met Pro Asn Ala Leu Ser Ala Leu Ser Asp Leu His Ala
        170                 175                 180

cat aaa ctg cgc gtt gac ccg gta aac ttc aag ctt ctg tct cat tgc      630
His Lys Leu Arg Val Asp Pro Val Asn Phe Lys Leu Leu Ser His Cys
    185                 190                 195

ctg ctg gtt act ctg gct gct cat ctg ccg gca gaa ttc act ccg gct      678
Leu Leu Val Thr Leu Ala Ala His Leu Pro Ala Glu Phe Thr Pro Ala
200                 205                 210                 215

gtt cat gct tct ctg gat aaa ttc ctg gct tct gtg tcg act gtt ctg      726
Val His Ala Ser Leu Asp Lys Phe Leu Ala Ser Val Ser Thr Val Leu
                220                 225                 230

act tct aaa tac cgc ggt gtt ctg tct ccg gca gac aaa act aac gtt      774
Thr Ser Lys Tyr Arg Gly Val Leu Ser Pro Ala Asp Lys Thr Asn Val
            235                 240                 245

aaa gct gct tgg ggt aaa gtt gga gct cat gct ggt gaa tac ggt gct      822
Lys Ala Ala Trp Gly Lys Val Gly Ala His Ala Gly Glu Tyr Gly Ala
        250                 255                 260

gaa gca ctc gag cgt atg ttc ctg tct ttc ccg act act aaa acg tac      870
Glu Ala Leu Glu Arg Met Phe Leu Ser Phe Pro Thr Thr Lys Thr Tyr
    265                 270                 275

ttc ccg cat ttc gac ctg taatgactgc ag                               900
Phe Pro His Phe Asp Leu
280                 285
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<210> SEQ ID NO 11
<211> LENGTH: 285
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 11

Met Ser His Gly Ser Ala Gln Val Lys Gly His Gly Lys Lys Val Ala
1 5 10 15

Asp Ala Leu Thr Asn Ala Val Ala His Val Asp Asp Met Pro Asn Ala
20 25 30

Leu Ser Ala Leu Ser Asp Leu His Ala His Lys Leu Arg Val Asp Pro
35 40 45

Val Asn Phe Lys Leu Leu Ser His Cys Leu Leu Val Thr Leu Ala Ala
50 55 60

His Leu Pro Ala Glu Phe Thr Pro Ala Val His Ala Ser Leu Asp Lys
65 70 75 80

Phe Leu Ala Ser Val Ser Thr Val Leu Thr Ser Lys Tyr Arg Gly Val
85 90 95

Leu Ser Pro Ala Asp Lys Thr Asn Val Lys Ala Ala Trp Gly Lys Val
100 105 110

Gly Ala His Ala Gly Glu Tyr Gly Ala Glu Ala Leu Glu Arg Met Phe
115 120 125

Leu Ser Phe Pro Thr Thr Lys Thr Tyr Phe Pro His Phe Asp Leu Ser
130 135 140

His Gly Ser Ala Gln Val Lys Gly His Gly Lys Lys Val Ala Asp Ala
145 150 155 160

Leu Thr Asn Ala Val Ala His Val Asp Asp Met Pro Asn Ala Leu Ser
165 170 175

Ala Leu Ser Asp Leu His Ala His Lys Leu Arg Val Asp Pro Val Asn
180 185 190

Phe Lys Leu Leu Ser His Cys Leu Leu Val Thr Leu Ala Ala His Leu
195 200 205

Pro Ala Glu Phe Thr Pro Ala Val His Ala Ser Leu Asp Lys Phe Leu
210 215 220

Ala Ser Val Ser Thr Val Leu Thr Ser Lys Tyr Arg Gly Val Leu Ser
225 230 235 240

Pro Ala Asp Lys Thr Asn Val Lys Ala Ala Trp Gly Lys Val Gly Ala
245 250 255

His Ala Gly Glu Tyr Gly Ala Glu Ala Leu Glu Arg Met Phe Leu Ser
260 265 270

Phe Pro Thr Thr Lys Thr Tyr Phe Pro His Phe Asp Leu
275 280 285

<210> SEQ ID NO 12
<211> LENGTH: 900
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 12

agatcttatt gattgatttc ctcttgttgt tggtacagag taccaaggcg agtccaattc 60

ccggtaccat tttttcaacg actgcgcaac tgattgcgac aacgagtaca actgctgtac 120

ggcttgcgag acaggcgaga cagtctagaa gtacgagtat ttgacgcgca actgggccat 180

ttgaagttcg aagacagagt aacggacgac caatgagacc gacgagtaga cggccgtctt 240

aagtgaggcc gacaagtacg aagagaccta tttaaggacc gaagacacag ctgacaagac 300

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tgaagattta tggcgccaca agacagaggc cgtctgtttt gattgcaatt tcgacgaacc 360

ccatttcaac ctcgagtacg accacttatg ccacgacttc gtgagctcgc atacaaggac 420

agaaagggct gatgattttg catgaagggc gtaaagctgg acagagtacc taggcgagtc 480

caatttccag taccattttt tcaacgactg cgcaactgat tgcgacaacg agtacaactg 540

ctgtacggct tgcgagacag gcgagacagt ctagaagtac gagtatttga cgcgcaactg 600

ggccatttga agttcgaaga cagagtaacg gacgaccaat gagaccgacg agtagacggc 660

cgtcttaagt gaggccgaca agtacgaaga gacctattta aggaccgaag acacagctga 720

caagactgaa gatttatggc gccacaagac agaggccgtc tgttttgatt gcaatttcga 780

cgaacccccat ttcaacctcg agtacgacca cttatgccac gacttcgtga gctcgcatac 840

aaggacagaa agggctgatg attttgcatg aagggcgtaa agctggacat tactgacgtc 900

<210> SEQ ID NO 13
<211> LENGTH: 62
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Cassette

<400> SEQUENCE: 13

ctagaataac taactaaagg agaacaacaa ccatgtctca tggttccgct caggttaaag 60

gt 62

<210> SEQ ID NO 14
<211> LENGTH: 62
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Cassette

<400> SEQUENCE: 14

ttattgattg atttcctctt gttgttggta cagagtacca aggcgagtcc aatttccagt 60

ac 62

<210> SEQ ID NO 15
<211> LENGTH: 108
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Cassette

<400> SEQUENCE: 15

tcgagcgcat gttcctgtct ttcccgacta ctaaaacgta cttcccgcat ttcgacctgg 60

gttctggtgg ttctcatgga tccgctcagg ttaaaggcca tggctgca 108

<210> SEQ ID NO 16
<211> LENGTH: 100
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Cassette

<400> SEQUENCE: 16

cgcgtacaag gacagaaagg gctgatgatt ttgcatgaag ggcgtaaagc tggacccaag 60

accaccaaga gtacctaggc gagtccaatt tccggtaccg 100

<210> SEQ ID NO 17

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<211> LENGTH: 1764
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: CDS
<222> LOCATION: (34)..(1752)

<400> SEQUENCE: 17

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tctagaataa ctaactaaag gagaacaaca acc atg tct cat ggt tcc gct cag      54
                                    Met Ser His Gly Ser Ala Gln
                                      1               5

gtt aag ggt cat ggt aaa aaa gtt gct gac gcg ttg act aac gct gtt      102
Val Lys Gly His Gly Lys Lys Val Ala Asp Ala Leu Thr Asn Ala Val
         10                  15                  20

gct cat gtt gac gac atg ccg aac gct ctg tcc gct ctg tca gat ctt      150
Ala His Val Asp Asp Met Pro Asn Ala Leu Ser Ala Leu Ser Asp Leu
     25                  30                  35

cat gct cat aaa ctg cgc gtt gac ccg gta aac ttc aag ctt ctg tct      198
His Ala His Lys Leu Arg Val Asp Pro Val Asn Phe Lys Leu Leu Ser
 40                  45                  50                  55

cat tgc ctg ctg gtt act ctg gct gct cat ctg ccg gca gaa ttc act      246
His Cys Leu Leu Val Thr Leu Ala Ala His Leu Pro Ala Glu Phe Thr
                 60                  65                  70

ccg gct gtt cat gct tct ctg gat aaa ttc ctg gct tct gtg tcg act      294
Pro Ala Val His Ala Ser Leu Asp Lys Phe Leu Ala Ser Val Ser Thr
             75                  80                  85

gtt ctg act tct aaa tac cgc ggt gtt ctg tct ccg gca gac aaa act      342
Val Leu Thr Ser Lys Tyr Arg Gly Val Leu Ser Pro Ala Asp Lys Thr
         90                  95                 100

aac gtt aaa gct gct tgg ggt aaa gtt gga gct cat gct ggt gaa tac      390
Asn Val Lys Ala Ala Trp Gly Lys Val Gly Ala His Ala Gly Glu Tyr
    105                 110                 115

ggt gct gaa gca ctc gag cgt atg ttc ctg tct ttc ccg act act aaa      438
Gly Ala Glu Ala Leu Glu Arg Met Phe Leu Ser Phe Pro Thr Thr Lys
120                 125                 130                 135

acg tac ttc ccg cat ttc gac ctg tct cat gga tcc gct cag gtt aaa      486
Thr Tyr Phe Pro His Phe Asp Leu Ser His Gly Ser Ala Gln Val Lys
                140                 145                 150

ggt cat ggt aaa aaa gtt gct gac gcg ttg act aac gct gtt gct cat      534
Gly His Gly Lys Lys Val Ala Asp Ala Leu Thr Asn Ala Val Ala His
            155                 160                 165

gtt gac gac atg ccg aac gct ctg tcc gct ctg tca gat ctt cat gct      582
Val Asp Asp Met Pro Asn Ala Leu Ser Ala Leu Ser Asp Leu His Ala
        170                 175                 180

cat aaa ctg cgc gtt gac ccg gta aac ttc aag ctt ctg tct cat tgc      630
His Lys Leu Arg Val Asp Pro Val Asn Phe Lys Leu Leu Ser His Cys
    185                 190                 195

ctg ctg gtt act ctg gct gct cat ctg ccg gca gaa ttc act ccg gct      678
Leu Leu Val Thr Leu Ala Ala His Leu Pro Ala Glu Phe Thr Pro Ala
200                 205                 210                 215

gtt cat gct tct ctg gat aaa ttc ctg gct tct gtg tcg act gtt ctg      726
Val His Ala Ser Leu Asp Lys Phe Leu Ala Ser Val Ser Thr Val Leu
                220                 225                 230

act tct aaa tac cgc ggt gtt ctg tct ccg gca gac aaa act aac gtt      774
Thr Ser Lys Tyr Arg Gly Val Leu Ser Pro Ala Asp Lys Thr Asn Val
            235                 240                 245

aaa gct gct tgg ggt aaa gtt gga gct cat gct ggt gaa tac ggt gct      822
Lys Ala Ala Trp Gly Lys Val Gly Ala His Ala Gly Glu Tyr Gly Ala
        250                 255                 260

gaa gca ctc gag cgt atg ttc ctg tct ttc ccg act act aaa acg tac      870
Glu Ala Leu Glu Arg Met Phe Leu Ser Phe Pro Thr Thr Lys Thr Tyr
```

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    265                 270                 275

ttc ccg cat ttc gac ctg ggt tct ggt ggt tct cat ggt tcc gct cag      918
Phe Pro His Phe Asp Leu Gly Ser Gly Gly Ser His Gly Ser Ala Gln
280                 285                 290                 295

gtt aag ggc cat ggt aaa aaa gtt gct gac gcg ttg act aac gct gtt      966
Val Lys Gly His Gly Lys Lys Val Ala Asp Ala Leu Thr Asn Ala Val
                300                 305                 310

gct cat gtt gac gac atg ccg aac gct ctg tcc gct ctg tca gat ctt     1014
Ala His Val Asp Asp Met Pro Asn Ala Leu Ser Ala Leu Ser Asp Leu
            315                 320                 325

cat gct cat aaa ctg cgc gtt gac ccg gta aac ttc aag ctt ctg tct     1062
His Ala His Lys Leu Arg Val Asp Pro Val Asn Phe Lys Leu Leu Ser
        330                 335                 340

cat tgc ctg ctg gtt act ctg gct gct cat ctg ccg gca gaa ttc act     1110
His Cys Leu Leu Val Thr Leu Ala Ala His Leu Pro Ala Glu Phe Thr
    345                 350                 355

ccg gct gtt cat gct tct ctg gat aaa ttc ctg gct tct gtg tcg act     1158
Pro Ala Val His Ala Ser Leu Asp Lys Phe Leu Ala Ser Val Ser Thr
360                 365                 370                 375

gtt ctg act tct aaa tac cgc ggt gtt ctg tct ccg gca gac aaa act     1206
Val Leu Thr Ser Lys Tyr Arg Gly Val Leu Ser Pro Ala Asp Lys Thr
                380                 385                 390

aac gtt aaa gct gct tgg ggt aaa gtt gga gct cat gct ggt gaa tac     1254
Asn Val Lys Ala Ala Trp Gly Lys Val Gly Ala His Ala Gly Glu Tyr
            395                 400                 405

ggt gct gaa gca ctc gag cgt atg ttc ctg tct ttc ccg act act aaa     1302
Gly Ala Glu Ala Leu Glu Arg Met Phe Leu Ser Phe Pro Thr Thr Lys
        410                 415                 420

acg tac ttc ccg cat ttc gac ctg tct cat gga tcc gct cag gtt aaa     1350
Thr Tyr Phe Pro His Phe Asp Leu Ser His Gly Ser Ala Gln Val Lys
    425                 430                 435

ggt cat ggt aaa aaa gtt gct gac gcg ttg act aac gct gtt gct cat     1398
Gly His Gly Lys Lys Val Ala Asp Ala Leu Thr Asn Ala Val Ala His
440                 445                 450                 455

gtt gac gac atg ccg aac gct ctg tcc gct ctg tca gat ctt cat gct     1446
Val Asp Asp Met Pro Asn Ala Leu Ser Ala Leu Ser Asp Leu His Ala
                460                 465                 470

cat aaa ctg cgc gtt gac ccg gta aac ttc aag ctt ctg tct cat tgc     1494
His Lys Leu Arg Val Asp Pro Val Asn Phe Lys Leu Leu Ser His Cys
            475                 480                 485

ctg ctg gtt act ctg gct gct cat ctg ccg gca gaa ttc act ccg gct     1542
Leu Leu Val Thr Leu Ala Ala His Leu Pro Ala Glu Phe Thr Pro Ala
        490                 495                 500

gtt cat gct tct ctg gat aaa ttc ctg gct tct gtg tcg act gtt ctg     1590
Val His Ala Ser Leu Asp Lys Phe Leu Ala Ser Val Ser Thr Val Leu
    505                 510                 515

act tct aaa tac cgc ggt gtt ctg tct ccg gca gac aaa act aac gtt     1638
Thr Ser Lys Tyr Arg Gly Val Leu Ser Pro Ala Asp Lys Thr Asn Val
520                 525                 530                 535

aaa gct gct tgg ggt aaa gtt gga gct cat gct ggt gaa tac ggt gct     1686
Lys Ala Ala Trp Gly Lys Val Gly Ala His Ala Gly Glu Tyr Gly Ala
                540                 545                 550

gaa gca ctc gag cgt atg ttc ctg tct ttc ccg act act aaa acg tac     1734
Glu Ala Leu Glu Arg Met Phe Leu Ser Phe Pro Thr Thr Lys Thr Tyr
            555                 560                 565

ttc ccg cat ttc gac ctg taatgactgc ag                              1764
Phe Pro His Phe Asp Leu
        570
```

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<210> SEQ ID NO 18
<211> LENGTH: 573
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 18

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Met Ser His Gly Ser Ala Gln Val Lys Gly His Gly Lys Lys Val Ala
  1               5                  10                  15

Asp Ala Leu Thr Asn Ala Val Ala His Val Asp Asp Met Pro Asn Ala
             20                  25                  30

Leu Ser Ala Leu Ser Asp Leu His Ala His Lys Leu Arg Val Asp Pro
         35                  40                  45

Val Asn Phe Lys Leu Leu Ser His Cys Leu Leu Val Thr Leu Ala Ala
     50                  55                  60

His Leu Pro Ala Glu Phe Thr Pro Ala Val His Ala Ser Leu Asp Lys
 65                  70                  75                  80

Phe Leu Ala Ser Val Ser Thr Val Leu Thr Ser Lys Tyr Arg Gly Val
                 85                  90                  95

Leu Ser Pro Ala Asp Lys Thr Asn Val Lys Ala Ala Trp Gly Lys Val
            100                 105                 110

Gly Ala His Ala Gly Glu Tyr Gly Ala Glu Ala Leu Glu Arg Met Phe
        115                 120                 125

Leu Ser Phe Pro Thr Thr Lys Thr Tyr Phe Pro His Phe Asp Leu Ser
    130                 135                 140

His Gly Ser Ala Gln Val Lys Gly His Gly Lys Lys Val Ala Asp Ala
145                 150                 155                 160

Leu Thr Asn Ala Val Ala His Val Asp Asp Met Pro Asn Ala Leu Ser
                165                 170                 175

Ala Leu Ser Asp Leu His Ala His Lys Leu Arg Val Asp Pro Val Asn
            180                 185                 190

Phe Lys Leu Leu Ser His Cys Leu Leu Val Thr Leu Ala Ala His Leu
        195                 200                 205

Pro Ala Glu Phe Thr Pro Ala Val His Ala Ser Leu Asp Lys Phe Leu
    210                 215                 220

Ala Ser Val Ser Thr Val Leu Thr Ser Lys Tyr Arg Gly Val Leu Ser
225                 230                 235                 240

Pro Ala Asp Lys Thr Asn Val Lys Ala Ala Trp Gly Lys Val Gly Ala
                245                 250                 255

His Ala Gly Glu Tyr Gly Ala Glu Ala Leu Glu Arg Met Phe Leu Ser
            260                 265                 270

Phe Pro Thr Thr Lys Thr Tyr Phe Pro His Phe Asp Leu Gly Ser Gly
        275                 280                 285

Gly Ser His Gly Ser Ala Gln Val Lys Gly His Gly Lys Lys Val Ala
    290                 295                 300

Asp Ala Leu Thr Asn Ala Val Ala His Val Asp Asp Met Pro Asn Ala
305                 310                 315                 320

Leu Ser Ala Leu Ser Asp Leu His Ala His Lys Leu Arg Val Asp Pro
                325                 330                 335

Val Asn Phe Lys Leu Leu Ser His Cys Leu Leu Val Thr Leu Ala Ala
            340                 345                 350

His Leu Pro Ala Glu Phe Thr Pro Ala Val His Ala Ser Leu Asp Lys
        355                 360                 365

Phe Leu Ala Ser Val Ser Thr Val Leu Thr Ser Lys Tyr Arg Gly Val
    370                 375                 380
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Leu Ser Pro Ala Asp Lys Thr Asn Val Lys Ala Ala Trp Gly Lys Val
385                 390                 395                 400

Gly Ala His Ala Gly Glu Tyr Gly Ala Glu Ala Leu Glu Arg Met Phe
                405                 410                 415

Leu Ser Phe Pro Thr Thr Lys Thr Tyr Phe Pro His Phe Asp Leu Ser
            420                 425                 430

His Gly Ser Ala Gln Val Lys Gly His Gly Lys Lys Val Ala Asp Ala
        435                 440                 445

Leu Thr Asn Ala Val Ala His Val Asp Asp Met Pro Asn Ala Leu Ser
    450                 455                 460

Ala Leu Ser Asp Leu His Ala His Lys Leu Arg Val Asp Pro Val Asn
465                 470                 475                 480

Phe Lys Leu Leu Ser His Cys Leu Leu Val Thr Leu Ala Ala His Leu
                485                 490                 495

Pro Ala Glu Phe Thr Pro Ala Val His Ala Ser Leu Asp Lys Phe Leu
            500                 505                 510

Ala Ser Val Ser Thr Val Leu Thr Ser Lys Tyr Arg Gly Val Leu Ser
        515                 520                 525

Pro Ala Asp Lys Thr Asn Val Lys Ala Ala Trp Gly Lys Val Gly Ala
    530                 535                 540

His Ala Gly Glu Tyr Gly Ala Glu Ala Leu Glu Arg Met Phe Leu Ser
545                 550                 555                 560

Phe Pro Thr Thr Lys Thr Tyr Phe Pro His Phe Asp Leu
                565                 570
```

<210> SEQ ID NO 19
<211> LENGTH: 1764
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 19

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agatcttatt gattgatttc ctcttgttgt tggtacagag taccaaggcg agtccaattc     60

ccggtaccat tttttcaacg actgcgcaac tgattgcgac aacgagtaca actgctgtac    120

ggcttgcgag acaggcgaga cagtctagaa gtacgagtat ttgacgcgca actgggccat    180

ttgaagttcg aagacagagt aacggacgac caatgagacc gacgagtaga cggccgtctt    240

aagtgaggcc gacaagtacg aagagaccta tttaaggacc gaagacacag ctgacaagac    300

tgaagattta tggcgccaca agacagaggc cgtctgtttt gattgcaatt tcgacgaacc    360

ccatttcaac ctcgagtacg accacttatg ccacgacttc gtgagctcgc atacaaggac    420

agaaagggct gatgattttg catgaagggc gtaaagctgg acagagtacc taggcgagtc    480

caatttccag taccattttt tcaacgactg cgcaactgat tgcgacaacg agtacaactg    540

ctgtacggct tgcgagacag gcgagacagt ctagaagtac gagtatttga cgcgcaactg    600

ggccatttga agttcgaaga cagagtaacg gacgaccaat gagaccgacg agtagacggc    660

cgtcttaagt gaggccgaca agtacgaaga gacctattta aggaccgaag acacagctga    720

caagactgaa gatttatggc gccacaagac agaggccgtc tgttttgatt gcaatttcga    780

cgaaccccat ttcaacctcg agtacgacca cttatgccac gacttcgtga gctcgcatac    840

aaggacagaa agggctgatg attttgcatg aagggcgtaa agctggaccc aagaccacca    900

agagtaccaa ggcgagtcca attcccggta ccatttttc aacgactgcg caactgattg    960

cgacaacgag tacaactgct gtacggcttg cgagacaggc gagacagtct agaagtacga   1020

gtatttgacg cgcaactggg ccatttgaag ttcgaagaca gagtaacgga cgaccaatga   1080
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gaccgacgag tagacggccg tcttaagtga ggccgacaag tacgaagaga cctatttaag   1140

gaccgaagac acagctgaca agactgaaga tttatggcgc cacaagacag aggccgtctg   1200

ttttgattgc aatttcgacg aaccccattt caacctcgag tacgaccact tatgccacga   1260

cttcgtgagc tcgcatacaa ggacagaaag ggctgatgat tttgcatgaa gggcgtaaag   1320

ctggacagag tacctaggcg agtccaattt ccagtaccat tttttcaacg actgcgcaac   1380

tgattgcgac aacgagtaca actgctgtac ggcttgcgag acaggcgaga cagtctagaa   1440

gtacgagtat ttgacgcgca actgggccat ttgaagttcg aagacagagt aacggacgac   1500

caatgagacc gacgagtaga cggccgtctt aagtgaggcc gacaagtacg aagagaccta   1560

tttaaggacc gaagacacag ctgacaagac tgaagattta tggcgccaca agacagaggc   1620

cgtctgtttt gattgcaatt tcgacgaacc ccatttcaac ctcgagtacg accacttatg   1680

ccacgacttc gtgagctcgc atacaaggac agaaagggct gatgattttg catgaagggc   1740

gtaaagctgg acattactga cgtc                                          1764
```

What is claimed is:

1. A heme protein, comprising a hemoglobin molecule including at least one circularly-permuted globin.

2. The protein of claim **1**, which is an oxygen-binding hemoglobin multimer.

3. The protein of claim **2**, wherein the hemoglobin multimer comprises crosslinked hemoglobin molecules each covalently linked to one another by a polypeptide having about three to about seven amino acids.

4. The protein of claim **3**, wherein each hemoglobin molecule is a crosslinked hemoglobin.

5. The protein of claim **4**, wherein the crosslinked hemoglobins include two genetic crosslinks.

6. The protein of claim **5**, which includes two hemoglobin molecules.

7. An oxygen-binding heme protein, comprising at least one hemoglobin molecule including two beta globins and a di-alpha globin construct, said di-alpha globin construct including a single polypeptide having a circularly-permuted alpha globin attached to another alpha globin by two genetic crosslinks.

8. The protein of claim **7**, comprising two or more of said hemoglobin molecules each attached to one another by a polypeptide linker covalently attaching termini of the circularly-permuted alpha globins.

9. The protein of claim **8**, wherein the polypeptide linker has about three to about seven amino acids.

10. The protein of claim **9**, wherein the genetic crosslink has one to about seven amino acids.

11. The protein of claim **7**, wherein the circularly permuted alpha globins have termini occurring in loop regions.

12. A polynucleotide encoding a circularly permuted globin.

13. The polynucleotide of claim **12** wherein the globin is alpha globin.

14. The polynucleotide of claim **12** which is a DNA sequence.

15. The polynucleotide of claim **14** which is a DNA sequence encoding a single polypeptide having a circularly-permuted alpha globin attached to another alpha globin by two genetic crosslinks.

16. The polynucleotide of claim **15**, which includes a DNA sequence sequentially encoding:

a first portion of a first, circularly-permuted alpha globin;

a first genetic crosslink;

a second alpha globin;

a second genetic crosslink; and

a second portion of the circularly permuted alpha globin, the first and second portions together constituting the entire circularly-permuted alpha globin.

17. A circularly-permuted alpha globin which assembles with another alpha and two beta globins to form an oxygen-carrying heme protein.

18. An isolated DNA sequence encoding a circularly-permuted alpha globin of claim **17**.

19. A vector including a polynucleotide sequence of claim **12**.

20. A host cell including and which expresses a DNA sequence of claim **12**.

21. A method for preparing an oxygen-binding heme protein, comprising culturing a host cell of claim **20**.

22. A method of increasing tissue oxygenation in a warm blooded animal, comprising administering to the animal a therapeutically-effective amount of a heme protein of claim **1** which binds oxygen.

23. A method of replacing hemoglobin in the bloodstream of a warm blooded animal, comprising administering to the animal an effective amount of a heme protein of claim **1** which binds oxygen.

24. A method of inducing vasoconstriction in a warm blooded animal, comprising introducing into the blood stream of the animal an effective amount of a heme protein of claim **1** which binds oxygen.

25. A method for increasing the oxygenation of an isolated organ or tissue, comprising contacting the organ or tissue with a heme protein of claim **1** which binds oxygen.

26. A pharmaceutical preparation, comprising a heme protein of claim **1** which binds oxygen, incorporated in pharmaceutically acceptable carrier.

27. A vector including a DNA sequence of claim **18**.

28. A host cell including and which expresses a DNA sequence of claim **18**.

* * * * *