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The Amino Acid Sequence of Canada Goose *(Branta canadensis)* and Mute Swan *(Cygnus olor)* Hemoglobins

Two Different Species with Identical β-Chains*

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Summary: The amino acid sequences of the α- and β-chains from the major hemoglobin component (HbA) of Canada goose *(Branta canadensis)* and mute swan *(Cygnus olor)* are given. The α-chains are of the α^A-type, since α^D-type was expressed but only found in low concentrations. By homologous comparison, greylag goose hemoglobin *(Anser anser)* and Canada goose hemoglobin α-chains differ by two exchanges, and β-chains by three exchanges. A valine substitution for threonine was found at position α34 (B15). This exchange is a result of a two point mutation. Thus, there are three nucleotide mutations in α-chains, as in β-chains. Substitutions in positions α34 (B15) and β125 (H3) have modified intersubunit contacts ($\alpha_1\beta_1$-contacts). A comparison of mute swan hemoglobin with greylag goose hemoglobin shows four exchanges in α-chains and three in β-chains. Canada goose and mute swan have identical β-chains, while α-chains differ in two amino acids. One of these exchanges is implicated in one of the $\alpha_1\beta_1$-contact points (α34) where isoleucine substitution for valine was found. Comparison of hemoglobins from different species in the same tribe (Anserini) shows a high homology between Canada goose and mute swan hemoglobins.

Die Aminosäuresequenz der Hämoglobine von Kanadagans (Branta canadensis) und Höckerschwan (Cygnus olor): Zwei verschiedene Arten mit identischen β-Ketten

Zusammenfassung: Die Primärstrukturen der α- und β-Ketten der Hauptkomponenten (HbA) von Kanadagans *(Branta canadensis)* und Höckerschwan *(Cygnus olor)* werden angegeben. Die α-Ketten sind vom α^A-Typ, der α^D-Typ wird ebenfalls exprimiert, aber nur in geringen Konzentrationen nachgewiesen. Beim homologen Vergleich mit dem Hämoglobin der Graugans *(Anser anser)* findet man bei der Kanadagans in den α-Ketten zwei, in den β-Ketten drei Unterschiede. Der Austausch α34 (B15) Thr→Val muß auf eine Zweipunktmutation zurückgeführt werden. Da-

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Abbreviations:
Quadrol = *N,N,N',N'*-tetrakis(2-hydroxypropyl)ethylenediamine; Tp = tryptic peptides; Reagent 1 = sodium 4-(isothiocyanato)benzenesulfonate; Reagent IV = trisodium 7-(isothiocyanato)-naphtalene-1,3,5-trisulphonate; TosPheCH_2Cl = chloro-(*N*-tosyl-L-phenylalanyl)methane; BG = barheaded goose; CG = Canada goose; CH = chicken; GG = greylag goose; PH = pheasant; SW = mute swan; inositol-P_5 = inositol-1,3,4,5,6-pentaphosphate;

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her haben sowohl in den α-Ketten als auch in den β-Ketten drei Mutationen stattgefunden. In α34 (B15) Val und β125 (H3) Asp sind $\alpha_1\beta_1$-Bindungsstellen verändert. Das Hämoglobin des Höckerschwans unterscheidet sich von dem der Graugans in den α-Ketten in vier, in den β-Ketten in drei Aminosäuren. Die β-Ketten von Kanadagans und Höckerschwan sind identisch. Die α-Ketten unterscheiden sich in zwei Aminosäuren. Eine Mutation findet man mit α34 (B15) Val→Ile an einer $\alpha_1\beta_1$-Bindungsstelle. Der Vergleich der Hämoglobine verschiedener Arten aus verschiedenen Gattungen der Gattungsgruppe Gänse (Anserini) ergibt die größte Homologie zwischen Kanadagans und Höckerschwan.

Key words: Hemoglobin, sequence, canada goose, mute swan.

Birds occupy an important position in biological evolution. A comparative study of the primary structure of bird hemoglobin and hemoglobin from other species is very interesting from the aspect of biochemical evolution and respiratory function. Studies of greylag goose *(Anser anser)* [2], barheaded goose *(Anser indicus)*[3,4] ostrich *(Struthio camelus)*[3], chicken *(Gallus gallus)*[5] and pheasant *(Phasianus colchicus colchicus)*[6] hemoglobins have already been published. In this paper we report the primary structures of Canada goose and mute swan hemoglobins A.

Materials and Methods

Isolation of hemoglobin

Blood was obtained from the major leg vein of mute swan and Canada goose. Erythrocytes were separated from the plasma by centrifugation and washed 3 times with physiological saline. Hemolysate was prepared by lysing the cells with distilled water. It was analysed by electrophoresis on polyacrylamide disc gels[7]; the results are shown in Fig. 1. Without prior separation of the hemoglobin components, globin was prepared from the whole hemolysate according to the method of Rossi-Fanelli and Antonini[8].

Separation of the chains

The native globin was separated into α- and β-subunit chains by chromatography on CM-Cellulose in 8M urea buffers in the pH range 5.0 to 5.8[9]. According to this method two α-chains (α^A and α^D) and one β-chain were isolated (Fig. 2). Between the two α-chains of the Canada goose an additional chain was found and degradated in the sequenator for 45 steps. The sequence was identical with the α^A-chain.

Furthermore, the polypeptide chains were resolved by polyacrylamide disc gel electrophoresis in presence of urea and Triton X-100 according to Alter et al.[10] (Fig. 3).

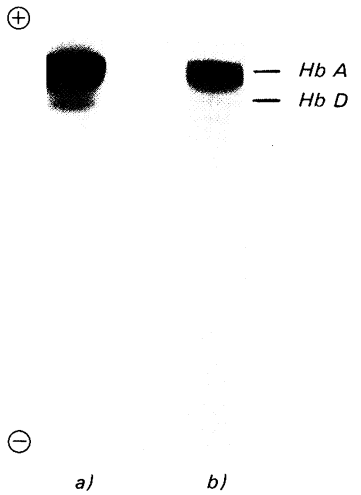

Fig. 1. Disc electrophoresis of a) Canada goose and b) mute swan hemoglobins.

Modification of the protein

Oxydation was carried out with performic acid[11], reduction with 2-mercaptoethanol and carboxymethylation with iodoacetamide[12].

Enzymatic digestion

Tryptic peptides of native globin were obtained by digestion with TosPheCH_2Cl-treated trypsin. Digestion was carried out at pH 9 to 10 for 4 h at room temperature. The peptides were first fractionated by gel filtration on Sephadex G-25 in 0.1M acetic acid. The single peaks were subsequently chromatographed on Dowex 50X4 42 °C with pyridine buffers and linear gradients (Fig. 4).

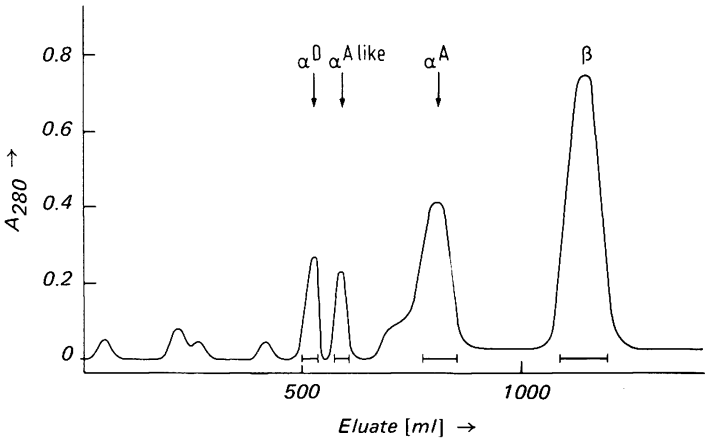

Fig. 2. Separation of the chains from Canada goose hemoglobin on a carboxymethyl-cellulose CM-52 column (2.5 x 12 cm) at a flow rate of 40 ml/h with a linear salt gradient.

Starting buffer: 8M urea + 0.02M sodium acetate + 0.02% 2-mercaptoethanol, pH 5.6. Final buffer: starting buffer + 0.4M NaCl pH 5.6. Fraction vol. 10 ml.

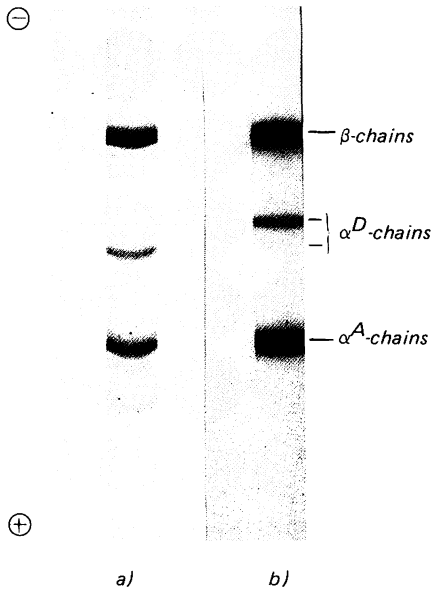

Fig. 3. Triton X-100 electrophoresis of a) Canada goose b) mute swan globins.

Asp-Pro cleavage

The cleavage of Asp-Pro bonds was carried out with 80% formic acid/6M guanidinium chloride solution at 42 °C for 60 h[13]. The peptides were then chromatographed on a Sephadex G-50 column in 8M urea/10% formic acid.

Amino acid analysis

Samples were hydrolysed in constant boiling hydrochloric acid at 110 °C for 20 h. The analyses were performed with a Beckman Model 121 C amino analyzer (Tables 1–5).

Sequence determination

Sequence determination was done by automatic Edman degradation[14] in a Beckman sequencer using Quadrol [14] and *N,N*-diethylaminopropyne[15] programs. The Quadrol programme was applied for sequencing the intact polypeptide chains, large peptides and lysine peptides which had been reacted with reagent I or IV[16,17]. The *N,N*-diethylaminopropyne programme was applied for sequencing arginine peptides.

Indentification of phenylthiohydantoin derivatives of amino acids

The derivatives were identified by thin-layer chromatography on silica gel (E. Merck, Darmstadt) using the solvent systems of Braunitzer et al.[18]. Some results were confirmed by high performance liquid chromatography [19]. The results of sequence determination corroborate the data of amino acid composition of the peptides and intact chains.

Results and Discussion

Polymorphism of Canada goose and mute swan hemoglobins

The red blood cells of the birds contain multiple types of hemoglobins which vary at different stages of development and during the life cycle. The hemoglobins found in early embrionic stages are called hemoglobins E, P, P' and M, in the late embryo HbH, and in the adult bird are called HbA and HbD[20].

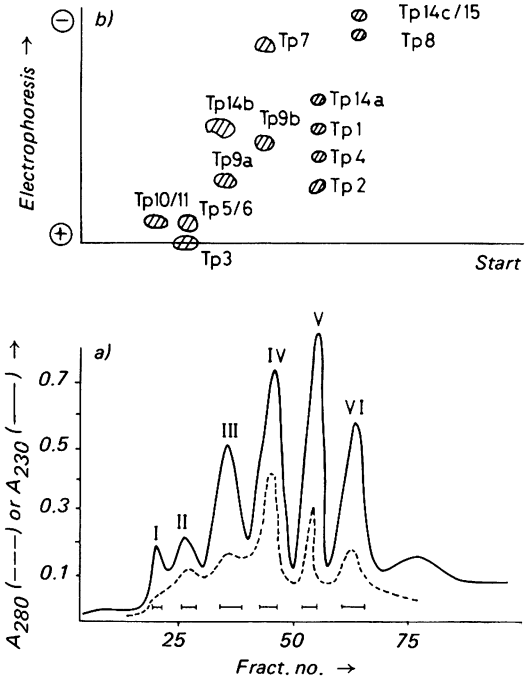

Fig. 4. a) Fractionation of β-chain soluble tryptic peptides from mute swan hemoglobin on a Sephadex G-25 (2.5 x 130 cm) at a flow rate 20 ml/h.
Buffer: 0.1M acetic acid. Fraction vol. 5 m*l*. b) The peptide map was obtained by high voltage electrophoresis.

Early embrionic fetal globin chains and adult globin chains are encoded by two developmentally regulated multigene families[21,22]. The two adult hemoglobins have different α-chains (α^A and α^D) and identical β-chains[20].

These two α-chains appear to be present in all birds, but the expression in different species differs. The α^D-gene is expressed in high levels in chicken and pheasant (about 20%) but in lower levels in geese and swan (about 5–10%). A larger difference in amino acid sequence was found between α^D- and α^A-chains of one species than between α^D-chains of different species[6]. This indicates that these globins are products of very old gene duplication. By homologous comparison of the same type of chains from different species, α^D-chains show a high, α^A-chains a normal and β-chains a reduced evolution rate.

We have analysed the partial sequence of hemoglobin D from Canada goose, and found that this amino acid sequence is very similar to the α^D-chains of chicken and pheasant hemoglobins (Fig. 5). The β-chains in the major and minor components are identical.

Physiology of Canada goose hemoglobin

In all birds inositol-P_5 is the major allosteric effector in hemoglobin[23,24]. The concentration

```
PH            Asn     Glu             Leu     Gln     Ala
CH                    Glu             Leu     Gln     Ala
CG   Met-Leu-Thr-Ala-Asp-Asp-Lys-Lys-Ile-Ile-Ala-Gln-Leu-Trp-Glu-
                       5                   10                  15

PH       Ala     Ser         Gln             Ala             Thr
CH       Ala     Ser         Gln             Ala             Val
CG  -Lys-Val-Ala-Gly-His-Gln-Asp-Glu-Phe-Gly-Asn-Glu-Ala-Leu-Glu-
                      20                  25                  30

PH               Thr-Ala
CH               Thr             Glu
CG  -Arg-Met-Phe-Val-Thr-Tyr-Pro-Gln-Thr-Lys-Thr-Tyr
                      35                  40
```

Fig. 5. Sequence of α^D-chains from hemoglobin D of Canada goose.
The sequence is homologously aligned with α^D-chains from pheasant[6] and chicken HbD[29].

Table 1. Canada goose hemoglobin, α^A-chains.
Amino acid composition of the soluble tryptic peptides.

	Tp5	Tp6	Tp7	Tp8	Tp9a	Tp9b	Tp10	Tp11	Tp14
Asx	–	1.02	–	–	2.98	1.00	–	2.01	–
Thr	1.11	0.78	–	–	–	–	–	–	–
Ser	–	1.03	–	–	0.87	0.96	–	–	–
Glx	1.00	1.99	–	–	0.95	1.02	–	–	–
Pro	0.85	0.90	–	–	–	–	–	0.72	–
Gly	–	0.99	1.00	–	0.94	–	–	–	–
Ala	1.02	1.01	1.03	–	5.29 (6)	0.98	–	–	–
Val	1.12	–	–	–	3.23	–	–	1.77	–
Ile	–	0.82	–	–	2.03	–	–	–	–
Leu	–	1.52 (1)	–	–	2.28	1.91	1.07	–	–
Tyr	0.88	0.89	–	–	–	–	–	–	0.98
Phe	0.92	1.94	–	–	–	–	–	0.87	–
Lys	1.02	1.05	1.00	1	1.37	1.07	–	0.93	–
His	–	2.03	0.97	–	1.03	1.04	–	–	–
Arg	–	–	–	–	–	–	0.93	–	1.02
Met	1.04	–	–	–	–	–	–	–	–
Cys	–	–	–	–	–	–	–	–	–
Sum.	9	16	4	1	21	8	2	7	2

Table 2. Canada goose hemoglobin, β-chains.
Amino acid composition of the soluble tryptic peptides.

	Tp5/6	Tp7	Tp8	Tp9a	Tp9b	Tp10/11	Tp14b	Tp14c/15
Asx	2.33	–	–	1.08	3.07	3.95	–	–
Thr	1.00	–	–	1.02	–	0.84	–	–
Ser	3.87	–	–	0.88	–	1.14	–	–
Glx	–	–	–	–	–	3.01	–	–
Pro	1.63	–	–	–	–	0.86	–	–
Gly	1.81	0.99	–	0.96	–	–	–	–
Ala	1.28	1.00	–	0.97	–	1.07	2.95	–
Val	1.27	–	–	1.78	–	0.87	1.16 (2)	–
Ile	1.03	–	–	–	0.92	–	–	–
Leu	2.52 (2)	–	–	1.21	1.04	3.05	1.42 (1)	–
Tyr	–	–	–	–	–	–	–	1.01
Phe	2.81	–	–	1.04	–	1.94	–	–
Lys	–	1.04	1	1.03	0.99	1.19	–	1.02
His	–	0.97	–	–	–	2.03	1.39 (1)	0.97
Arg	1.30 (1)	–	–	–	–	1.00	1.05	–
Met	0.90	–	–	–	–	–	–	–
Cys	–	–	–	–	–	1.01	–	–
Sum.	21	4	1	10	6	22	8	3

Table 3. Mute swan hemoglobin, α^A-chains.
Amino acid composition or the soluble tryptic peptides.

	Tp1	Tp2	Tp3	Tp4	Tp5	Tp6	Tp7	Tp8	Tp9a	Tp9b	Tp10	Tp11	Tp14
Asx	1.07	1.03		1.97		0.96			2.96	1.16		2.41	
Thr		0.98		1.06	1.21	0.88			1.14				
Ser	1.00		1.00			0.90			1.23	1.09			
Glx				2.02	1.15	1.93				1.17			
Pro					1.05	0.90						0.79	
Gly			0.98	2.84		1.20	1.10		1.22				
Ala	1.92			1.89	0.90	1.15	1.13		5.84	1.15			
Val	1.03	1.02	0.92						2.89			1.81	
Met					0.89								
Ile				0.94	0.93	0.86			1.81				
Leu	0.99			1.03		0.98			2.00	2.21	0.87		
Tyr				0.91	0.96	0.88							0.98
Phe			0.95		0.92	1.88						0.84	
Lys	0.97	0.98	1.14		0.98	1.22	0.90	1	1.23	1.00		0.89	
His				1.31		2.22	0.86		1.15	1.00			
Arg				1.00							1.12		1.01
Sum.	7	4	5	15	9	16	4	1	21	8	2	7	2

Table 4. Mute swan hemoglobin, β-chains.
Amino acid composition of the soluble tryptic peptides.

	Tp1	Tp2	Tp3	Tp4	Tp5/6	Tp7	Tp8	Tp9a	Tp9b	Tp10/11	Tp14a	Tp14b	Tp14c/15
Asx			2.16		2.13			1.02	2.76	3.85			
Thr	1.12	1.17		1.00	1.01			0.95		0.83			
Ser					3.78			0.89		0.97			
Glx	1.99	1.13	1.01	1.15						2.60			
Pro				1.07	2.12					0.87			
Gly		2.03	1.30		2.09	0.98		1.05					
Ala	0.99		3.89		1.26	1.07		1.05		1.25		3.26	
Val	0.95		1.74	0.78	1.06			1.81		1.28	0.96	1.48 (2)	
Met					1.06								
Ile		0.96		0.76	0.95				1.02				
Leu		2.00	1.19	2.18	2.14			1.03	1.19	2.86	1.05	1.12	
Tyr				1.02									0.83
Phe					2.99			0.93		1.73			
Lys	1.03	0.89				0.98	1	0.98	1.09	1.12			1.00
His	0.86					0.97				2.08		1.06	1.19
Arg			0.97	1.08	1.00					0.93	0.99	1.27	
Cys			0.87							1.01			
Trp	1.00	1.00		1.00									
Sum.	8	9	13	10	21	4	1	10	6	22	3	8	3

Table 5. Canada goose hemoglobin. Amino acid composition of the complete α^A- and α^D-chains and of the *C*-terminal peptides HP-2 after hydrolytic cleavage (Asp-Pro bond).

TH = Determination after total hydrolysis; SA = sequence determination; values in brackets indicate the differences in comparison to the grayleg goose.

	α^A-Chains				β-Chains				α^D-Chains
	Complete chains		HP-2 peptide		Complete chains		HP-2 peptide		Compl. chains
	TH	SA	TH	SA	TH	SA	TH	SA	TH
Asx	11.30	11 (+1)	2.30	2	14.73	15 (+2)	4.30	4 (+2)	12.52
Thr	7.57	8 (−1)	2.77	3	6.47	7 (+1)	0.94	1	5.33
Ser	6.97	7	1.91	2	6.02	6 (−1)	–	–	6.77
Glx	9.30	9 (−1)	1.19	1	10.29	10 (−2)	3.14	3 (−2)	13.94
Pro	4.24	5	2.53	3	5.34	5	1.98	2	5.05
Gly	8.78	9	2.12	2	7.90	8	1.10	1	7.03
Ala	19.02	19	5.32	5	17.07	17	7.51	8	15.55
Val	14.65	15 (+1)	6.55	7	11.85	12	3.95	4	10.49
Ile	4.50	5	1.02	1	6.61	7	2.86	3	3.33
Leu	14.50	14	6.09	6	17.68	18	5.97	6	15.32
Tyr	3.75	4	1.00	1	2.20	2	1.01	1	4.31
Phe	8.07	8	3.48	4	8.14	8	3.00	3	7.18
Lys	12.00	12	3.20	3	10.13	10	3.16	3	10.29
His	9.61	10	3.52	4	7.17	7	2.69	3	6.11
Arg	2.91	3	1.00	1	5.86	6	3.05	3	3.94
Met	1.38	1	–	–	1.05	1	–	–	2.55
Cys	1.95	2	2.11	2	4.10	4	0.94	1	
Trp	–	–	–	–	2.73	3	0.85	1	
Sum.		141		47		146		47	141

of inositol-P_5 in Canada goose erythrocytes is 5.2μM [25], which is 20% higher than in greylag goose (4.3μM) and barheaded goose (4.5μM). In addition low levels of ATP (0.6μM) and 2,3-diphosphoglycerate (0.3μM) are present[25]. The concentration of ATP in Canada goose erythrocytes is about 30% higher than in erythrocytes of barheaded goose and 10% higher than in greylag goose red blood cells. The $p(O_2)_{50}$ for the whole blood of Canada goose is 5.6 kPa indicating a lower oxygen affinity compared to that of the greylag goose. The $p(O_2)_{50}$ estimate for geese hemoglobin without phosphate is 0.1–0.3 kPa, but is increased in the presence of phosphate. It was found that the highest influence of phosphate is in Canada goose red blood cells. This result correlates with the high concentration of inositol-P_5 and other phosphates and also with mutations in the primary structure of the chains. We suggest that substitution in $\alpha_1\beta_1$-contact sites may account for higher influence of inositol-P_5 on Canada goose hemoglobin.

Table 6. Comparison of amino acid and nucleotide substitutions in the hemoglobins of grayleg goose (GG), barheaded goose (BG), Canada goose (CG) and mute swan (SW).

	Amino Acid α/β	Nucleotide α/β
CG/SW	2/0	2/0
GG/BG	3/1	3/1
CG/GG	2/3	3/3
SW/GG	4/3	4/3
CG/BG	5/2	6/2
SW/BG	7/2	7/2

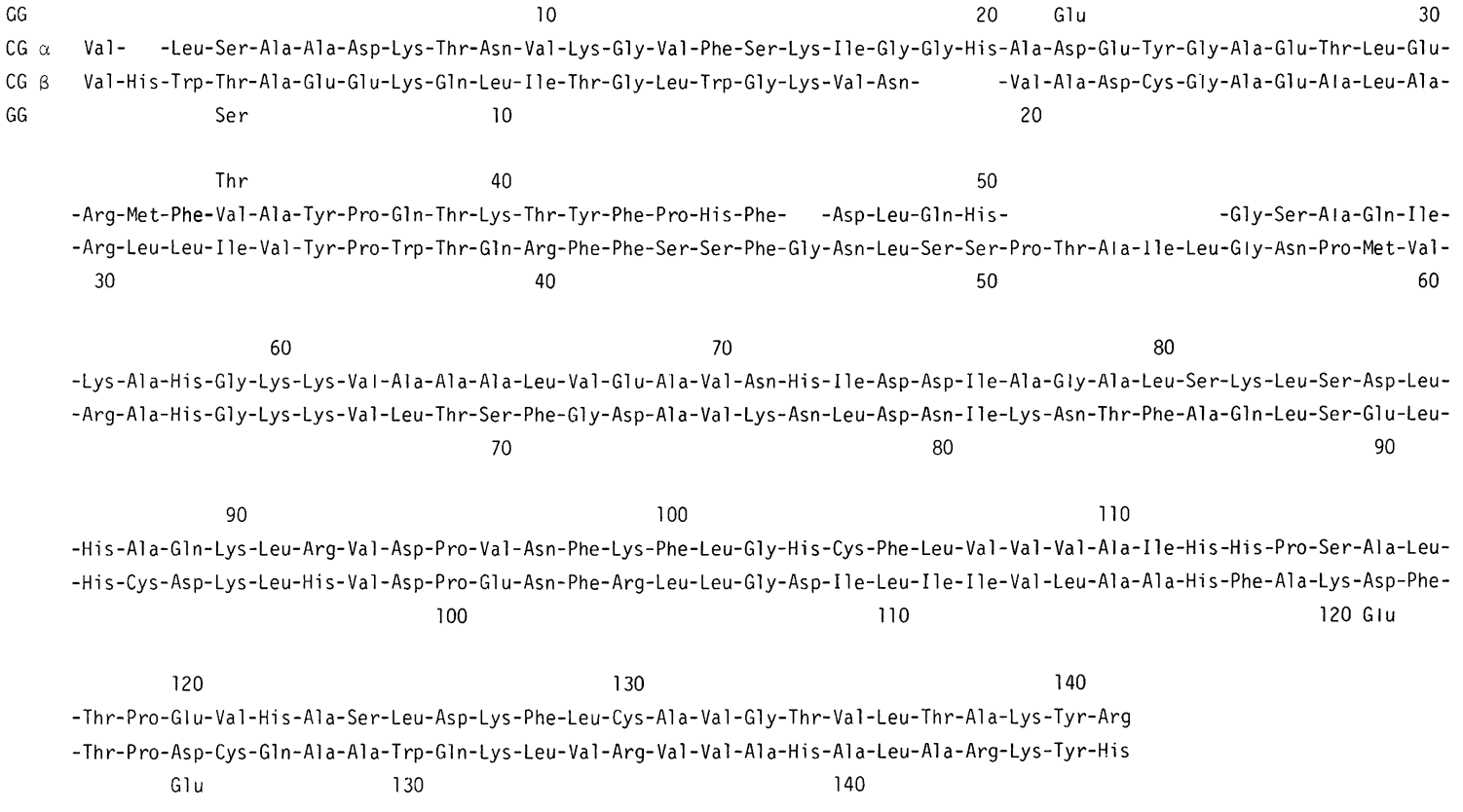

Fig. 6. Sequence of α^{A}- and β-chains from hemoglobin A of Canada goose. The sequence is homologously aligned with greylag goose hemoglobin[2].

```
                                                  10                                  20
GG α                                                                                  Glu-Glu
SW α   Val-   -Leu-Ser-Ala-Ala-Asp-Lys-Thr-Asn-Val-Lys-Gly-Val-Phe-Ser-Lys-Ile-Gly-Gly-His-Ala-Asp-Asp-Tyr-Gly-Ala-Glu-Thr-Leu-
SW β   Val-His-Trp-Thr-Ala-Glu-Glu-Lys-Gln-Leu-Ile-Thr-Gly-Leu-Trp-Gly-Lys-Val-Asn-       -Val-Ala-Asp-Cys-Gly-Ala-Glu-Ala-Leu-
GG β                Ser
                                                  10                                  20

        30                                        40                                  50
GG α                    Thr
SW α   -Glu-Arg-Met-Phe-Ile-Ala-Tyr-Pro-Gln-Thr-Lys-Thr-Tyr-Phe-Pro-His-Phe-   -Asp-Leu-Gln-His-                    -Gly-Ser-Ala-
SW β   -Ala-Arg-Leu-Leu-Ile-Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe-Phe-Ser-Ser-Phe-Gly-Asn-Leu-Ser-Ser-Pro-Thr-Ala-Ile-Leu-Gly-Asn-Pro-
GG β
             30                                   40                                  50

                                  60                                  70                                  80
GG α
SW α   -Gln-Ile-Lys-Ala-His-Gly-Lys-Lys-Val-Ala-Ala-Ala-Leu-Val-Glu-Ala-Val-Asn-His-Ile-Asp-Asp-Ile-Ala-Gly-Ala-Leu-Ser-Lys-Leu-
SW β   -Met-Val-Arg-Ala-His-Gly-Lys-Lys-Val-Leu-Thr-Ser-Phe-Gly-Asp-Ala-Val-Lys-Asn-Leu-Asp-Asn-Ile-Lys-Asn-Thr-Phe-Ala-Gln-Leu-
GG β
             60                                   70                                  80

                                  90                                  100                                 110
GG α
SW α   -Ser-Asp-Leu-His-Ala-Gln-Lys-Leu-Arg-Val-Asp-Pro-Val-Asn-Phe-Lys-Phe-Leu-Gly-His-Cys-Phe-Leu-Val-Val-Val-Ala-Ile-His-His-
SW β   -Ser-Glu-Leu-His-Cys-Asp-Lys-Leu-His-Val-Asp-Pro-Glu-Asn-Phe-Arg-Leu-Leu-Gly-Asp-Ile-Leu-Ile-Ile-Val-Leu-Ala-Ala-His-Phe-
GG β
             90                                   100                                 110

                                  120                                 130                                 140
GG α                                                                                  Thr
SW α   -Pro-Ser-Ala-Leu-Thr-Pro-Glu-Val-His-Ala-Ser-Leu-Asp-Lys-Phe-Leu-Cys-Ala-Val-Gly-Ala-Val-Leu-Thr-Ala-Lys-Tyr-Arg
SW β   -Ala-Lys-Asp-Phe-Thr-Pro-Asp-Cys-Gln-Ala-Ala-Trp-Gln-Lys-Leu-Val-Arg-Val-Val-Ala-His-Ala-Leu-Ala-Arg-Lys-Tyr-His
GG β          Glu            Glu
            120                                  130                                 140
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Fig. 7. Sequence of α^A- and β-chains from hemoglobin A of mute swan. The sequence is homologously aligned with greylag goose hemoglobin[2].

Comparison of Canada goose hemoglobin with greylag goose hemoglobin shows five exchanges (Fig. 6). Two of these substitutions are in $\alpha_1\beta_1$-contacts but only one [α34 (B15), Thr→Val] could make a significant contribution to the elevated influence of inositol-P_5 on Canada goose hemoglobin. Substitution at position β125 (H3, Glu→Asp) was also found in barheaded goose hemoglobin.

Physiology of mute swan hemoglobin

Investigations on the affinity of oxygen for hemoglobin of mute swan are not reported here. Bech and Johnsen[26] measured ventilation and gas exchange in this hemoglobin, and from their data we have estimated the $p(O_2)_{50}$ for mute swan whole blood to be 5.1 kPa. This value is lower than that reported for Canada goose and therefore indicates a higher oxygen affinity. A high concentration of inositol-P_5 or Ile-substitution for Val at position α34 may be responsible for elevated oxygen affinity of mute swan hemoglobin.

Classification and biology of Canada goose and mute swan

The Canada goose belongs to the genus *Branta*, the mute swan to the genus *Cygnus*. Both genera belong to the family Anatidae. The Canada goose is the most common goose in North America and gives rise to the most subspecies. They inhabit low lying areas. Mute swans inhabit Europe and middle Asia. Mute swans differ from geese in their larger size and longer necks. Of the tribe Anserini (geese) which consists of four genera, the primary structures of hemoglobins from genus *Cygnus*, *Anser* and *Branta* have already been published. Table 6 shows the lowest number of mutations between Canada goose and mute swan α- and β-chains of both species are identical. According to homologous comparison of hemoglobins, Canada goose and mute swan must be closely related, although they belong to two different tribes. This could be mean that classification from biological data should be revised or that the real genetic distance between these two species is not apperent because of their parallel evolution. A close relationship between *Cygnus* and *Branta* is also indicated by the comparative analysis of lysozymes. Most avian species possess either the goose type or chick type of lysozyme in their egg white. In contrast, the Australian black swan *(Cygnus astratus)* and Canada goose have mixture of both types of lysozymes[27,28].

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