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STRUCTURAL AND FUNCTIONAL CHARACTERIZATION OF A CANINE MINOR GLYCOXYLATED HEMOGLOBIN, HB CAN A1c

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ABSTRACT

Structural and functional studies of minor glycosylated hemoglobin in non-human mammals have been very few. Therefore, we attempted further characterization of a canine glycosylated hemoglobin (tentatively designated as Hb Can A1c) which had been isolated by Bio-Rex 70 chromatography (Enoki, Y. et al. (1982) Hemoglobin 6, 143-151). After isolation of the constituent subunits by hybridization-mercuration technique, we found by peptide mapping that the structural modification was confined in aminotermini of the β subunits in which glycosyl ketoamine linkage was also shown to be localized by thiobarbituric acid reaction. Compared with the major hemoglobin (Hb Can A0), Hb Can A1c was characterized by such functional properties as a slightly lower oxygen affinity and a markedly reduced response to the allosteric effects of carbon dioxide and organic phosphates. The anion and H+ Bohr effects were not different from those in Hb Can A0. All these facts support that this hemoglobin is a canine counterpart of Hb A1c.

Presence of several minor hemoglobins in normal human blood was first reported by Allen et al (1). One of these hemoglobins, Hb A1c, has not only been investigated in more detail but clinically used as an informative monitor for long-term control of blood glucose in diabetics (2,3). Later, this hemoglobin was re-

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vealed to derive from the major hemoglobin, Hb A0, by its post
translational glycosylation at amino-termini of the β chains. McDonald and her colleagues developed a chromatographic proce-
to isolate each of the glycosylated hemoglobins Hb A1a-1, A1a
A1b and A1c(7), which enabled them to study their structures and functions in further detail(7,8). Recently further isolations
characterizations of the minor hemoglobins were conducted usin
similar but improved procedures (9,10). We also studied some
structural and functional features of hemoglobins A1a, A1b and
A1c and found that their structural differences were confined
solely to the β subunits and their altered functions could be
interpreted by assuming their allosteric structures to be fixed
more or less "T" state(11,12).

Relatively few studies, however, have been reported on the
two kinds of hemoglobins in non-human mammals, which would be inte-
esting in both basic and clinical concerns (13-16). We recent
ly described a procedure to separate a minor glycosylated hemoglo-
bin (Hb Can A1a-2) from canine blood, which could be considered as
d a canine counterpart of Hb A1c (17). We report here further struc-
tural and functional studies of this hemoglobin (Hb Can A1c).

MATERIALS AND METHODS

Heparinized blood was obtained by venipuncture from normal
human adults or healthy mongrel dogs. Blood was processed to
obtain destromatized hemolysate and stored in carboxy form in
an ice bath.

Isolation of Hb Can A1c: We employed the previously reported
chromatographic procedure using Bio-Rex 70 (Bio-Rad, 200-400 mesh
(17). Purity of the isolated protein was assured by gel electro-
phoresis after concentrating it with an ultrafiltration apparat
(Toyo, UK-10). The protein was then de-ionized by passing throu
a column of Bio-Rad mixed bed exchanger AG 501X8(50) and stored
in carboxy form on ice.

Preparation of native α and β subunits from Hb Can A1c: The
mercurated subunits were prepared by our modification of the
Bucci-Fronticelli method (18, 19), demercurated with 8-mercapto-
ethanol on a Sephadex G-10 column (2.5 x 30 cm) (20), deionized,
and finally concentrated by ultrafiltration. Care was taken to
always keep the hemoglobins saturated with carbon monoxide.

Electrophoresis: Starch gel technique was performed with Tris-
EDTA-borate buffer system (pH 8.6) in the cold (21). The gel
slices were stained with 10% Amido Black 10B.

Peptide mapping analysis: The isolated subunits were treated with
200 molar excess of sodium borohydride in 0.1 M bis-Tris buffer
(pH 7.5) for 1 h at 10°C, followed by exhaustive dialysis against
CO-saturated deionized water. The globins were then prepared by
an acid-acetone method (22) and aminoethylated by the procedure of
Ratery and Cole (23). After incubation of the aminoethylated
globins with one hundredth by weight of trypsin (Worthington, X3
crystallized) in 0.2 M ammonium bicarbonate buffer (pH 8.0) at
37°C for 5 h, the tryptic digests were lyophilized. Two mg of the
digests were peptide-mapped on Whatman 3MM filter paper (37 x 60
cm), first electrophoresis in one direction (3,000 V-60 min, 10°C)
followed by the ascending chromatography at 20°C for 18 h in the
direction vertical to the first electrophoresis. Compositions of
the developer systems were pyridine/acetic acid/water (1:1:114 by
vol., pH 4.7) for the electrophoresis and butanol/acetic acid/
water/pyridine for the chromatography (15:3:12:10 by vol.). The
dried maps were stained by spraying ninhydrin reagent or appro-
priate specific stain reagents.

Phosphorus assay: The dry ashing procedure of Ames and Dubin (24)
was employed.

Thiobarbituric acid reaction: The procedure of Flückiger and
Winterhalter (13) was generally followed after treatment of the
protein with 0.3 M oxalic acid at 100°C for 5 h. The positive
reaction was characterized by an absorption maximum at 443 nm.

Oxygen equilibrium studies: We followed a spectrophotometric pro-
cedure which has been routinely used in this laboratory (25).
The measurements were usually performed in 0.05 M bis-Tris buffer
at 20°C, unless otherwise stated. Final concentrations of Cl−
FIGURE 1
Separation of mercurated $\beta^\text{Can Aic}$ and $\alpha^\text{Ao}$ chains from mercurated hybrid ($\alpha^\text{Ao} \beta^\text{Can Aic}$) carboxyhemoglobin on CM 32 (3 x 10 cm). Hb, 508 mg; fraction vol., 7 ml. pH profile (---).

were adjusted to 0.035 M with NaCl in bis-Tris or Tris buffers, when used for determinations of the Bohr effect, and to 0.1 M for determinations of the organic phosphate effects. The specific effect of carbon dioxide (carbamate effect) was studied at constant pH of 7.3 in CO$_2$-HCO$_3$\textsuperscript{-} buffer, the ionic strength being adjusted to 0.1 with NaCl. Hemoglobin solution in a calculated concentration of bicarbonate-NaCl was first deoxygenated by alternating evacuations and N$_2$ flushings, followed by rotation in a water bath (20°C). After spectroscopic assurance of the complete deoxygenation ($A_{555}/A_{540} > 1.24$), a calculated amount of carbon dioxide was injected into the tonometer to give a desired CO$_2$ tension and consequently, the desired pH. After reconfirmation of the complete deoxygenation, the usual procedure for the oxyg
Starch gel electrophoresis of $\alpha$ and $\beta$ chains of Hb Can AIC and Hb Can AO. TEB buffer (pH 8.6), Amido Black 10B stain. The left of each pair represents the run with the subunit treated with 200 molar in excess of NaBH$_4$.

equilibrium measurement was followed (25). The result, in 0.05 M bis-Tris buffer (pH 7.3 and ionic strength 0.1), was taken as the one in the absence of carbon dioxide.

Chemicals used were all of reagent grade and supplied from Nakarai Chemicals (Kyoto).

RESULTS

Native $\beta$Can AIC subunit was separated on a cation exchanger CM 32 (Whatman) by our hybridization-mercuration technique (18) from the $\alpha_2$$\beta_2$Can AIC hybrid hemoglobin which was prepared from human adult major hemoglobin (Hb A0) and Hb Can AIC (Fig. 1). Native $\alpha$Can AIC was similarly obtained on an anion exchanger DE 32 (Whatman) from the $\alpha_2$Can AIC,$\beta_2$A0 hybrid hemoglobin (18). Figure 2 shows the electrophoretic comparison of the isolated constituent subunits of Hb Can A0 and Hb Can AIC. We could conclude from the results that the enhanced anodal mobility of Hb Can AIC (17) was caused solely by the $\beta$ subunits. This finding was completely consistent with our previous result by the acid hybridization method (17). It should also be noted that no alteration was induced
in the electrophoretic behavior of $\beta^{\text{Can A1c}}$ by the treatment with sodium borohydride.

Fig. 3 (bottom) shows the thiobarbituric acid reaction of the constituent subunits of Hb Can A1c which had been shown to be positive for the reaction(17). The positive reaction was found only for the $\beta$ subunits, indicating that glycosyl ketamine linkage was confined there. It should also be noted that like Hb Can A0, Hb Can A1-1, probably a mixture of minor hemoglobins with faster
Peptide maps of tryptic digests of aminoethylated $\beta$Can A0 (top) and $\beta$Can A1c (bottom). Arrows indicate the position of the normal tryptic peptide I and the shaded spot indicates the modified position.

Anodic mobilities than Hb Can A1c(17), exhibited no reaction (Fig. 3-Top). Amino acid compositions of $\beta$Can A0 and $\beta$Can A1c were little different from each other (data not shown). All these results strongly suggest that like human Hb A1c, Hb Can A1c would be derived from Hb Can A0 by glycosylation in the $\beta$ subunits. No trace of phosphorus was found in both $\alpha$ and $\beta$ subunits of Hb Can A1c.
Intrinsic oxygen equilibrium curves of Hb Can A0 (○) and Hb Can A1c (●). 0.05 M bis-Tris (pH 7.3, 0.01 M Cl\(^-\)), 20°C Hb / ml. Log P\(_{50}\)'s for Hb Can A0 and Hb Can A1c were 0.229 and 0.256, respectively.

The site of the glycosylation was then searched for by a mapping method (Fig. 4). The peptide maps of β\(^\text{Can A0}\) and β\(^\text{Can A1c}\) were different in that first, the tryptic peptide I observed in the former was lacking in the latter, and secondly, instead, a slow, very slowly staining His-positive peptide spot appeared in position a little anodic to the normal position. Since the no tryptic peptide I can be shown to constitute the octapeptide including the N-terminal valine, Val-His-Leu-Thr-Ala-Glu-Glu-Lys; these results were consistent with a notion that location of structural modification might involve the N-terminus of the β chains.

Hb Can A1c in 0.1 M phosphate (pH 7.0) manifested a single and symmetrical boundary in sedimentation velocity analysis at the \(s_{20,w}^0\) was estimated as 4.5S which was not different from that for Hb Can A0. Weight-average molecular weight of the hemoglobin in 0.05 M bis-Tris buffer (pH 7.3) was \(6.54 \cdot 10^4\) by equilibrium sedimentation and it was essentially identical to \(6.47 \cdot 10^4\) for Hb Can A0.
Fig. 5 shows the intrinsic oxygen equilibrium curves of stripped Hb Can A1c and Hb Can A0 under the least influence of allosteric effectors, i.e., in 0.05 M bis-Tris buffer containing 0.01 M Cl\(^-\) (pH 7.3). A slightly but distinctly lower oxygen affinity should be noted in Hb Can A1c compared with Hb Can A0, although the cooperativity was little different from each other (Hill's exponent 2.8). The Bohr effects of both hemoglobins were essentially identical when observed in 0.05 M bis-Tris and constant Cl\(^-\) concentration of 0.035 M (Fig. 6). The Bohr factor, \(\Delta \log P_{50}/\Delta \text{pH}\), calculated in pH range from 7.0 to 7.8 was -0.63 for Hb Can A1c and -0.62 for Hb Can A0.

As shown in Fig. 7, both the hemoglobins were indistinguishable as to the anion (Cl\(^-\)) effect on the oxygen affinity. This finding was rather different than the situation in human Hb A1c.
FIGURE 7
Anion (Cl\textsuperscript-) effect of Hb Can A\textsuperscript{0} (○) and Hb Can A\textsubscript{1c} (●). 0.05 M bis-Tris (pH 7.3), 20°C, 3 mg Hb/ml. Ordinate: Increment in log P\textsubscript{50} upon increment of NaCl (abscissa). Dotted curves were the results with the human corresponding molecular species, Hb and Hb A\textsubscript{1c}.

TABLE I. Effect of organic phosphates on the oxygen equilibrium of Hb Can A\textsuperscript{0} and Hb Can A\textsubscript{1c}.

<table>
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<th>DPG mM</th>
<th>Hb Can A\textsubscript{1c}</th>
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<th>DPG mM</th>
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0.05 M bis-Tris-0.1 M Cl\textsuperscript-, pH 7.3, 20°C. DPG, supplied as cyclohexylammonium salt, was converted to free acid and titrated to pH 7.3 with NaOH.
which exhibited a considerably depressed anion (Cl⁻) effect compared with Hb A₀ (Enoki, unpublished). The effect of organic phosphates upon the oxygen affinity is summarized in Table 1. It was evident that the glycosylated hemoglobin showed a remarkable decrease in the effect as compared with Hb Can A₀, the relative extent of the reduction being 73 % for 2,3-diphosphoglycerate (DPG) at 2 mM and 87 % for inositol hexaphosphate (IHP) at 1 mM. The specific CO₂ (carbamate) effect is shown in Fig. 8. In this effect too, Hb Can A₁c showed an enormous reduction (77 % at PCO₂ 80 torr) compared with Hb Can A₀. This depression was a little more than that for the human counterpart, Hb A₁c (68 % relative to Hb A₀) under comparable conditions (Enoki, unpublished).

**DISCUSSION**

Hb A₁c and the other minor hemoglobins have attracted wide attention not only in their clinical concerns with diabetes
mellitus(2,3) but also in a basic interest that they represent a rather rare occasion of natural hemoglobins with lowered oxygen affinity present in normal human blood(6,8,11). Presence of minor hemoglobins in non-human blood seems highly probable in the light of recent mounting evidence (15,16) but there have been no reports of structural or functional studies. Recently, we developed a procedure using Bio-Rex 70 chromatography to isolate one of minor hemoglobins, Hb Can \text{AI}_c, from canine hemolysates and reported a partial characterization of the protein(17). In this paper we describe the results of further characterization of Hb Can \text{AI}_c from which we concluded that this hemoglobin was the canine counterpart of Hb \text{AI}_c with abnormal oxygenation properties, a little different from those of human Hb \text{AI}_c.

A body of experimental evidence (Figs. 3 and 4) strongly suggests that like human Hb \text{AI}_c(4-6) the chemical nature of a structural modification in Hb Can \text{AI}_c may be the attachment of a sugar moiety via ketoamine linkage to the NH$_2$-terminal amino groups of the normal $\beta$ chains. This is completely consistent with our anticipation made in the previous study(17). By such a modification the protein may be endowed with a reduction in the positive net charge which results in a higher anodic mobility (Fig. 2).

Furthermore, the functional properties observed here, especially the depressed CO$_2$ effect, strongly support the idea that the sugar attaches to the NH$_2$-terminal amino groups of the $\beta$ chains in Hb Can \text{AI}_c. Compared with Hb Can \text{A}0, a minor but distinct reduction in the intrinsic oxygen affinity and marked depression of the DPG or IHP effect and CO$_2$ effect are observed (Figs. 5 and Table 1). The responses to anion ($\text{Cl}^-$) and $\text{H}^+$ are the same as those in Hb Can \text{A}0 (Figs. 6 and 7). All these functional properties are qualitatively very similar to those in human Hb \text{AI}_c except for the unaltered $\text{Cl}^-$ effect in Hb Can \text{AI}_c.

When a chemical modification is introduced in the primary structure of hemoglobin, functional changes might be induced in two ways, the one localized and the other more wide-spread.
human minor hemoglobins including Hb A1c, for example, formation of
the sugar adducts with the B chain NH2-terminal groups results in
1) abolishment of the reactivity of the termini(8,11) which have
been shown to constitute the bulk, or a part of, the binding sites for
molecular CO2(27) and organic phosphates(28) and also 2) a dampened
R-T excursion of the quaternary structure upon oxygenation(11,12).
The present results of a series of the functional alterations
(Fig. 8 and Table I) may be mainly due to the first aspect of the
functional consequences. Similar findings were reported for human
Hb A1c(6,11). The array of positively charged residues between
the B chains in the central cavity of hemoglobin (B1 Val, B2 His,
B82 Lys, B143 His) has been considered to repel each other in a low
anion concentration, and thus destabilizes the T-state quaternary
conformation of deoxyhemoglobin relative to the R-state confor-
mation of oxyhemoglobin(29). A slight but distinct reduction in
the intrinsic oxygen affinity (Fig. 5) can be explained by the elim-
ination of the positive charges of the NH2-termini with the sugar
adduct formation, leading to relative stabilization of the deoxy-
hemoglobin structure. Since the B-chain terminal amino groups
have been generally considered not to be involved in oxygen-linked
binding of anions and H+ to hemoglobin(30), it will be expected
that we could not find any differences in both anion (Cl-) and Bohr
effects between Hb Can A0 and Hb Can A1c (Figs. 6 and 7). The
effect of CO2 on the oxygenation of hemoglobin has been ascribed
to the oxygen-linked binding of the molecule with the unprotonated
a-amino groups of the constituent subunits(27). In normal human
hemoglobin, the contribution by the B chains is shown to be more
predominant, constituting 60 to 70% of the whole effect (27,31).
A reduction by 68% in the CO2 effect of human Hb A1c, as shown
in Fig. 8, is therefore a result well compatible with the previous
results. The present result with Hb Can A1c (Fig. 8) may reflect
a possible species difference, as the contribution by the B chains
is 77% and is a little more than in human hemoglobin. However,
a possibility still exists, that in Hb Can A1c, the structural
modification may extend some influence upon the reactivity
the untouched amino terminus of the α chains.

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