TURNOVER OF AMINO-TERMINAL ACETYLATED AND NON-ACETYLATED HEMOGLOBINS *IN VIVO*[*]

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SUMMARY: The significance of amino terminal acetylation in the turnover of hemoglobin was analyzed by measuring the synthesis and degradation of hemoglobins A and B in domestic cat blood. The two hemoglobins occur as mixtures in cat blood and are structurally very similar except for β-chain amino terminal acetylation found in HbB which is chiefly responsible for their difference in isoelectric pH. The hemoglobins were labeled by administering radioactive amino acids to anemic animals and their specific radioactivities were monitored at intervals for several days thereafter until the average life span of the erythrocytes was exceeded. The results showed that the turnover of hemoglobin is unrelated to isoelectric pH or to amino-terminal acetylation of the protein.

It has become apparent recently that amino-terminal acetylation is a common phenomenon among proteins in eukaryotic cells and questions have been raised with increasing frequency concerning its physiological significance. At present, however, there is no information on the functional properties of such proteins that can be ascribed directly to amino-terminal acetylation except for what is known about the feline acetylated hemoglobins. The β-chain amino-terminals in one of these hemoglobins are acetylated, and as a consequence, these proteins fail to undergo functional interaction with

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allosteric effectors such as 2,3-DPG,[1] ATP and IHP (1-3). That binding of organic phosphates by hemoglobin requires positively charged free β-chain amino terminals has been demonstrated unequivocally (1, 3-6).

Concerning other possible consequences of amino-terminal acetylation, Jornvall (7) has suggested that it may be a general mechanism for stabilization of proteins aganist proteolytic degradation, and Brown and Roberts (8) have reported recently that their preliminary experiments with L-cells suggest a lower turnover rate for proteins containing acetylated amino-terminal residues than for those with free amino-terminals. However, data supporting these conclusions have not been published as yet. The hemoglobin system of domestic cat blood seems to be of special advantage to examine this question since it is comprised of mixtures of acetylated (HbB) and non-acetylated (HbA) hemoglobins with structures that are very similar to one another except for the presence of β-chain amino-terminal acetylation in HbB (3,9). The amount of the acetylated component among these animals is variable, ranging from as little as 5-10% to as much as 50% of the total hemoglobin; in fact, the acetylated component can comprise as much as 90-95% of the hemoglobin in certain other members of the Felidae (10). The presence of hemoglobins with amino-acetylated β-chains as quantitatively major components of blood is unusual and can be exploited to examine the question of stability of these proteins *in vivo*. The experiments reported here were conducted to determine the turnover of domestic cat HbA and HbB following incorporation of labeled amino acids into these proteins in animals made anemic by phenylhydrazine administration. The data obtained indicate that the two hemoglobins have the same turnover rate *in vivo*.

MATERIALS AND METHODS

Labeling of hemoglobins in vivo. Cats possessing 1/1 or 2.3/1 (HbA/HbB) phenotypes were made anemic by daily injections of a 2.5% neutralized phenylhydrazine solution (0.25 ml/kg) until the hematocrit was about 18% and

[1] The abbreviations used are: 2,3-DPG, 2,3-diphosphoglycerate; IHP, inositol hexaphosphate.

the reticulocyte count 50%. 1.0 mCi of neutralized tritiated amino acid mixture, or a neutralized mixture of 50 μCi of [^{14}C]glycine and 50 μCi [^{14}C]-valine in isotonic saline was then injected intravenously (day 0). Samples of blood were obtained by venipuncture at intervals thereafter until the analysis indicated nearly complete turnover of the labeled hemoglobins. [2-^{14}C]glycine, L-[^{14}C(U)]valine, and tritiated L-amino acid mixtures (NET-250) were purchased from New England Nuclear.

Analysis of blood and hemoglobins. Blood samples were collected in heparin. Hematocrits were determined by the micro-capillary technique and reticulocyte counts were obtained using peripheral smears stained with new methylene blue (11). Plasma was removed by centrifugation and cells washed three times with normal saline. The cells were lysed by addition of 1.5 volumes of cold distilled water, and the stroma was removed by centrifugation. Hemoglobin concentration was determined by the cyanmethemoglobin method (12) or from the absorbance at 415 and 540 nm. The relative amounts of the two major cat hemoglobin components in the blood was determined by gel isoelectric focusing as previously described (2, 13).

Lysates were bubbled with CO for 15-30 seconds to convert the hemoglobins to their CO derivatives. HbA and HbB were separated on 0.9 x 20 cm columns of Bio-Rex 70 (200-400 mesh) resin equilibrated at 4° with 0.05 M phosphate buffer (pH 6.5) containing 1 mM EDTA and eluted with the same buffer at a flow rate of 1 ml/hr. After HbB was completely eluted, HbA was removed with 0.1 M phosphate buffer, pH 6.8. Where indicated, the acid-acetone procedure was used to separate heme from globin (14). The globin was washed twice with cold acetone and once with ethyl ether. Heme was recovered by evaporation of the acid-acetone and further purified by washing with 0.1 N HCl after redissolving the samples in minimal amounts of acetone.

Counting of radioactivity. Samples (0.5 ml) of the eluant from the Bio-Rex 70 column were transferred into vials containing 10 ml of Aquasol (New England Nuclear) for counting. Globin preparations were counted after solubilization with Protosol (New England Nuclear). Specific activity of heme samples was determined by dissolving the samples in acetone, plating on aluminum planchets, and counting in a gas-flow counter.

RESULTS

The phenotype of the cats used in this work was determined by isoelectric focusing to be 1/1 or 2.3/1, i.e., HbB comprised about 50% or 30% of the total hemoglobin. HbB, the acetylated component, has the greater anodic mobility (2). As indicated in Table 1, the difference in isoelectric pH for the two proteins can be accounted for entirely by the presence or absence of the acetyl blocking group at the β-chain terminus. The three amino acid differences do not alter the charge of the protein but the amino terminal acetylation in β^B results in the net loss of two positive charges in the HbB tetramer, giving it a lower isoelectric pH.

Figure 1 presents data on changes in certain hematological parameters monitored during the course of *in vivo* labeling of the hemoglobins of a 1/1 phenotype cat. The data show that the relative proportions of HbA/HbB

Table I

Differences in Structure Between β^A and β^B of Cat Hemoglobins A and B

Position	β^A	β^B
1	Gly	N-Acetyl-Ser
139*	Asn	Ser
144	Lys	Arg

*Substitution established from amino acid compositions of the respective tryptic peptides.

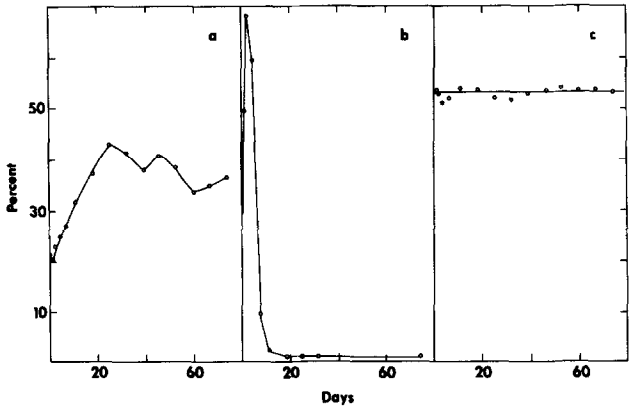

Figure 1. Hematologic parameters monitored during *in vivo* labeling experiment on a cat of 1/1 (HbA/HbB) phenotype. a. Hematocrit; b. reticulocyte count; c. percent HbA.

remained constant throughout the experiment. It is possible to resolve both HbA and HbB into additional components (15) and to demonstrate anemia related changes in their relative amounts within HbA and HbB fractions. However, this does not affect the results or conclusions of this work since the sum of components in these fractions remains constant and all components in HbB are acetylated and all components in HbA are not.

After injection of labeled amino acids, the specific radioactivities of both hemoglobins increased rapidly and at similar rates (Figure 2). Furthermore, both HbA and HbB attained approximately the same maximum specific

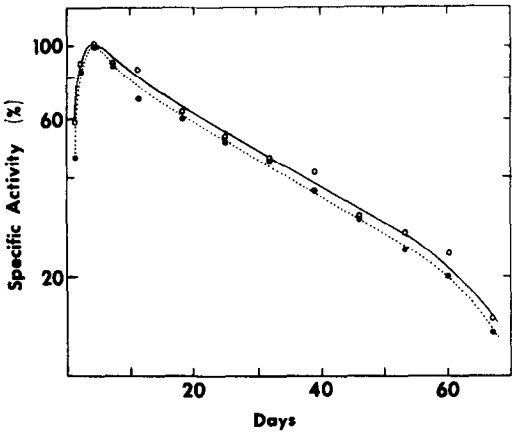

Figure 2. Specific radioactivities of cat hemoglobins after injection of tritiated amino acid mixture into a cat of 1/1 (HbA/HbB) phenotype. o—o HbB, ●·····● HbA. Maximum specific activities were 4180 and 4090 dpm/mg for HbB and HbA respectively.

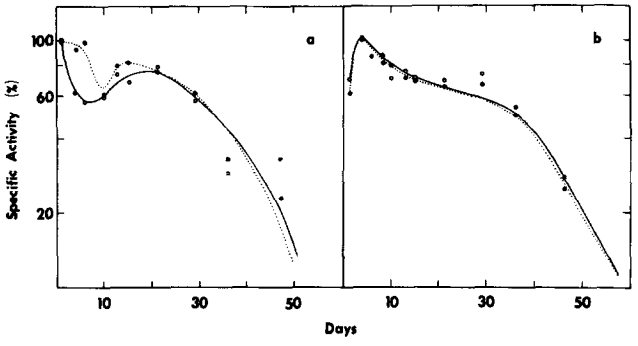

Figure 3. Specific radioactivities of cat hemoglobins after injection of [^{14}C]glycine and [^{14}C]valine into a cat of 2.3/1 (HbA/HbB) phenotype. o—o HbB, ●·····● HbA. a. Specific activity of heme; maxima 54 and 48 cpm/A_{557} for HbB and HbA respectively; b. specific activity of globin; maxima 1980 and 1990 dpm/mg for HbB and HbA respectively.

activity, and demonstrated nearly identical rates of decline in specific activity. Similar results were obtained with an animal of 2.3/1 phenotype (Figure 3). It can be seen again that the two hemoglobins acquired similar specific radioactivities at identical rates and turned over at the same rates when measured in terms of either globin or of hemin. The fact that the two hemoglobins rapidly attained the same specific radioactivity even though the

circulating non-radioactive pool of HbA was 2.3 times that of HbB at the time of administration of the pulse of labeled amino acids indicates that the former is synthesized effectively 2.3 fold faster than HbB.

DISCUSSION

The involvement of minor modifications in turnover of serum proteins and of erythrocytes is well known. Morell *et al.* (16) and Lenten and Ashwell (17) demonstrated that enzymatic desialyation of serum proteins resulted in their rapid removal from circulation by the liver through recognition by hepatocyte surface receptors. Subsequently, Jancik and Schauer (18) demonstrated that treatment of rabbit erythrocytes with neuraminidase decreased their lifetime presumably by modification of membrane glycoproteins which may be recognized by elements of the reticuloendothelial system when exposed. If amino terminal protein acetylation is a similar process, it would appear to be a simpler form which might be an evolutionary precursor of the more sophisticated glycoprotein system.

Several tables of amino terminal acetylated proteins have been published in recent years (7, 19-21). Although none is complete, together they demonstrate that the phenomenon is ubiquitous and that serine and alanine tend to be the amino terminal residues most commonly acylated. Of the proteins known to be acetylated at the amino terminus, the cat hemoglobins provide one of the more convenient systems for studying the suggestions of Jornvall (7) and of Dice and Goldberg (22) that acetylation and pI are important in determining the *in vivo* protein half-life. Consideration of cat HbB and HbA indicates, however, that the difference in their isoelectric points makes no difference whatsoever in their *in vivo* stability. Furthermore, if indeed acetylation is meant to stabilize the protein it would not be consistent with the supposed direct relationship between higher isoelectric point and stability of proteins (22) since amino-acetylation causes the protein to shift to a lower rather than higher isoelectric point.

In evaluating the present results, several considerations arise. First, the β^A terminal residue is glycine while the β^B residue is serine. Perhaps in the absence of the acetyl group this difference would render HbA slightly more stable than HbB. With no satisfactory means of deacetylating HbB *in vivo* or *in vitro*, this possibility cannot be evaluated at present. Also, it may be objected that the erythrocyte is an atypical cell in that it is incapable of protein synthesis and hence does not turnover proteins in a manner similar to hepatocytes or other somatic cells. However, other species are well known to have erythrocytic endopeptidases which are specific for amino terminal residues (23,24), so the potential for recognition of amino-terminal differences in protein sequence may exist in red cells. Recently, however, Roberts and Yuan have provided evidence that proteins acetylated with acetic anhydride in tissue culture have the same half-life as proteins that are not acetylated (25).

The erythrocyte contains other proteins that are acetylated at the amino terminus (21). These include carbonic anhydrases, superoxide dismutase, and phosphoglycerate kinase. Since most species possess both an acetylated and non-acetylated carbonic anhydrase, this protein would provide another convenient test of the Jornvall and the Dice and Goldberg hypotheses.

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