Misincorporation of Alkylated Amino Acids into Hemoglobin – a Possible Source of Background Alkylations

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Misincorporation of 2-hydroxyethylated amino acids into hemoglobin during *de novo* synthesis was studied by injecting mice with radiolabelled *N*-(2-hydroxyethyl)valine, *S*-(2-hydroxyethyl)cystine or N^{π}-(2-hydroxyethyl)histidine. The results showed that *S*-(2-hydroxyethyl)cysteine and N^{π}-(2-hydroxyethyl)histidine were misincorporated, whereas *N*-(2-hydroxyethyl)valine was not. Monitoring of *in vivo* doses of hydroxyethylating agents by determination of *N*-(2-hydroxyethyl)valine was free of the disturbing influence of such misincorporation.

The determination of alkylated amino acids in hemoglobin has been used to monitor tissue doses in experimental animals after exposure to various alkylating agents (for reviews see Refs. 1 and 2) and in man after occupational exposure to ethylene oxide[3] and propylene oxide.[4] The analytical methods used are based on GC/MS analysis after hydrolysis of the protein, isolation of the alkylated products followed by derivatization[5] or cleavage of the N-terminal amino acid (valine in hemoglobin) in the form of a pentafluorophenylthiohydantoin.[6,7] These methods are sensitive enough to determine levels of alkylated products that occur in the protein for "natural" reasons.

In connection with the development of the methods to monitor exposure to ethylene oxide and propylene oxide, histidine adduct levels of the order of 0.1–1 nmol per g globin were recorded for hydroxyethyl in samples from individuals without known exposure to hydroxyethylating agents and levels of around 0.1 nmol g^{-1} hydroxypropyl were found in a few subjects.[8] The corresponding levels of *N*-(2-hydroxyethyl)valine[7] and *S*-(2-hydroxyethyl)cysteine (unpublished results) were 0.03–0.8 nmol and 1–3 nmol per g globin, respectively. Comparatively high levels of methylated cysteine[9] and histidine have been determined in animals and man.

Electrophilic reagents react with nucleophilic groups in their environment in a way which is random in the sense that, although reaction rates vary depending on the properties of the reactants (the electrophile and the nucleophilic counterpart), no specificities of an either/or character occur. This means that an electrophile that reacts with nucleophilic sites in hemoglobin will also react with nucleophilic sites – critical or uncritical – in the DNA. The determination of hemoglobin adducts may therefore be applied as a sensitive chemical endpoint in risk identification, provided it can be ascertained that the adducts are introduced into the hemoglobin *in vivo* through electrophilic reactions. The aim of the present study was to investigate the possibility that misincorporation of alkylated amino acids, which may be present in foods for various reasons (*vide infra*), during *de novo* synthesis of hemoglobin* contributes to the background levels found in unexposed individuals.

Materials and methods

N-([^{3}H]2-Hydroxyethyl)-DL-valine (spec. act. 30.0 Ci $mmol^{-1}$) was synthesized according to Calleman.[10] *S*-(2-Hydroxyethyl)-[^{14}C]DL-cysteine

*The same type of preparation of the globin as used for monitoring purposes is also used in the present work.[3,4]

(spec. act. 24.2 mCi mmol^{-1}) was synthesized according to Zilkha and Rappoport[11] and N^{τ}-([^{14}C]2-hydroxyethyl)-L-histidine (spec. act. 22 mCi mmol^{-1}) was synthesized according to Calleman and Wachtmeister.[12] All products were purified on a Dowex® 50 W × 4 ion exchange column and the syntheses were verified by TLC. The corresponding unlabelled amino acids were synthesized according to the same methods and their structures were verified by mass fragmentography.

Male CBA mice, 9–15 weeks old (wt 22–25 g) were used in all experiments. Alkylated amino acids were dissolved in saline and given each separately to two mice by i.p. injection. The animals were sacrificed after 6 days (mice injected with *S*-(2-hydroxyethyl)cysteine after 4 days) and blood was collected in heparinized tubes. The red cells were spun down and washed 4 times with saline and lysed by adding distilled water. The hemoglobin was then applied on a Sephadex G-25 column, eluted with 10 mM formic acid and fractions were collected. The hemoglobin fractions were dialysed against water for 2 h and lyophilized. The hemoglobin was dissolved in water and globin isolated by precipitation with 1 % HCl in acetone according to Anson and Mirsky.[13] Globin samples (156–178 mg) were hydrolyzed with 6 M HCl in evacuated tubes at 120 °C for 17 h. Previous studies[14] have demonstrated a high recovery of hydroxyethylated amino acids by this procedure. The hydrolysates were evaporated to dryness, dissolved in water and incubated at 50 °C for 30 min to make sure that no 2-chloroethyl amino acids remained in the hydrolysates. Unlabelled *N*-(2-hydroxyethyl)valine, *S*-(2-hydroxyethyl)cysteine and N^{τ}-(2-hydroxyethyl)histidine were added (about 5 mg) to the respective samples and the samples chromatographed on a Dowex® 50 W × 4 ion exchange column. The column was eluted with 1 M HCl followed by 2 M HCl. Amino acids in the fractions were detected and identified by TLC and radioactivity in the fractions was measured.

The samples from mice injected with *S*-(2-hydroxyethyl)cysteine and N^{τ}-(2-hydroxyethyl)histidine showed an increased radioactivity in corresponding fractions but the samples from mice injected with *N*-(2-hydroxyethyl)valine did not. Fractions containing *S*-(2-hydroxyethyl)cysteine and N^{τ}-(2-hydroxyethyl)histidine were evaporated and esterified with 1.25 M HCl/MeOH and

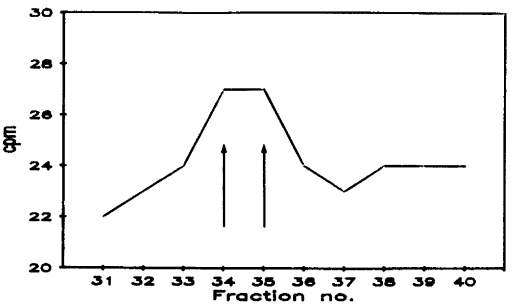

Fig. 1. Radioactivity per fraction obtained after Animex A-5 ion exchange chromatography of *S*-(2-hydroxyethyl)cysteine methyl ester. The arrows show the fractions in which the carrier, *S*-(2-hydroxyethyl)cysteine methyl ester, was eluted.

chromatographed on an Aminex® A-5 ion exchange column from which fractions were collected. Methyl esters of *S*-(2-hydroxyethyl)cysteine (Fig. 1) and N^{τ}-(2-hydroxyethyl)histidine were detected and identified by TLC and radioactivity in the corresponding fractions was measured. From earlier experience with similar separations, the recovery of isolated quantities was estimated to be about 30 %.

Results and discussion

The results (see Table 1) show that *S*-(2-hydroxyethyl)cysteine and N^{τ}-(hydroxyethyl)histidine are misincorporated during *de novo* synthesis of hemoglobin*, whereas *N*-(2-hydroxyethyl)valine is not. *N*-alkylated valine is not likely to be incorporated into proteins, since the nitrogen of valine is involved in the peptide linkages between amino acids. This concerns the N-terminal amino acid as well, the synthesis of mammalian proteins being initiated by a methionine.[15]

The error frequency in protein synthesis has been estimated at about 3 parts per 10,000 with respect to valine substitution for leucine[16,17] (similar values have, e.g., been estimated for cysteine substitution for arginine or tryptophan, cf. Refs. 18 and 19). Generally, aminoacyl-tRNA synthetases exhibit a much lower specificity towards amino acids that are not normally present in cells.[20] As was first shown by Loftfield *et al.*[21] alloisoleucine has some 100 times higher competition ratios than natural amino acids for valine

*See previous footnote.

Table 1. Misincorporation of alkylated amino acids into mouse hemoglobin after a single injection of radiolabelled compounds

Amino acid	Amount of globin analyzed (mg)	Injected amount (dpm g^{-1} body weight) A	Incorporation into globin (dpm g^{-1}) B	$(B/A) \cdot 10^6$ (95 % confidence interval)
N-(2-hydroxymethyl)-valine	90	$2.7 \cdot 10^{9a}$	<50	<0.02[b]
S-(2-hydroxyethyl)-cysteine	168	$2.2 \cdot 10^8$	256	1.2 (±0.4)
N^t-(hydroxyethyl)-histidine	178	$7.3 \cdot 10^8$	450	0.6 (±0.06)

[a]Refers to the amount of the L-enantiomer.
[b]Upper 95 % confidence limit of difference between three fractions which would have contained 80 % of the *N*-(2-hydroxyethyl)valine if it had been present, and three control fractions made up by liquid (1 M HCl) from fractions eluting before the front. Each fraction was counted for 200 min. Adjustment was made for variations between counted vessels with respect to background values; these were determined by counting for 100 min before and 100 min after the experimental countings.

and isoleucine. This might be partly a consequence of the "proof-reading" exerted by the hydrolytic capacity of aminoacyl-tRNA synthetase[22] directed towards tRNAs mischarged by natural amino acids and inefficient for tRNA complexes of unnatural amino acids, as has been shown for the action of the Val enzyme on *O*-methylthreonine-$tRNA^{Val}$.[23] To the extent that chemically changed amino acids appear in cells and provided coupling to some tRNA is possible, considerable misincorporation of unnatural amino acids is possible.

Certain alkylated amino acids may be at hand in foods for several reasons. Hydroxyethylated amino acids may be formed in crop organisms by hydroxyethylating agents such as ethylene oxide known to be generated endogenously in both plants[24] and animals.[25] Foods may also have been fumigated with ethylene oxide[26] or exposed to this reagent from tobacco smoke.[27] It is further quite possible that alkylated amino acids are formed in food preparation at elevated temperatures. The formation of hydroxyethylated amino acids in this way was so far not investigated, however. It seems important to determine the degree of hydroxyethylation (and levels of other adducts) in food proteins to estimate the contribution of foodstuffs to the background levels of hydroxyethylated (and also other types of alkylated) amino acids in hemoglobin.

Other possible sources of the observed background of hydroxyethylations are, for instance: exposure to ethene in the environment (ethene is metabolically converted to ethylene oxide[28]) and endogenous formation of ethene (e.g., in lipid peroxidation[29] or through metabolism of methionine[30]).

The absence of incorporation of *N*-(2-hydroxyethyl)valine into hemoglobin makes it plausible that the determination of alkylation of N-terminal valine reflects reaction of electrophilic reagents at this position. This is an advantage of the monitoring method based on N-terminal alkylvaline determination.[6,7]

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