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CYSTEINE ADDUCTS OF HUMAN HAEMOGLOBIN MEASURED BY ISOELECTRIC FOCUSING IN POLYACRYLAMIDE GELS WITH A NON-LINEAR pH GRADIENT

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SUMMARY

The in vitro formation of adducts from human haemoglobin formed by alkylation with methylmethanesulphonate, dimethyl sulphate and iodoacetamide was determined with isoelectric focusing in ultra-thin polyacrylamide gels with a non-linear pH gradient. The most important adduct seen in the gels was identified as HbA alkylated at the β-93 cysteine. Influences of the chemical nature of the alkylating agents and of the biological environment are discussed. The method is suggested to be applicable to monitoring the biological effects of low, long-term exposure to mixtures of alkylating agents or of exposure to unknown compounds.

INTRODUCTION

Since the suggestion by Ehrenberg et al. [1] in 1974 of the use of in vivo alkylation of human haemoglobin (Hb) as a dose monitor for low, long-term exposure to genotoxic agents, reaction products for over 30 radiolabelled alkylating agents with haemoglobin have been measured in animal studies [2,3]. As a result of the difficulties encountered in the development of analytical methods with the necessary sensitivity, data on humans are still limited. Although methods for the determination of the products of methylating agents [4,5] and of some aromatic compounds [6–8] have been described, practical results are limited to the products of ethylene oxide [9,10] and propylene oxide [11].

Despite the large differences in the analytical methods that have been used, all the methods developed so far follow the same general scheme. They start with degradation (sometimes partial) of the alkylated haemoglobin, and continue with purification of the products. As a result of this last feature they have a high selectivity towards the alkylating moiety. In all cases, with the possible exception of

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the modified Edman degradation technique for the determination of alkylated N-terminal valine [12], only adducts of substances that are specifically looked for are detected. Although this certainly is an advantage with respect to the sensitivity of the methods, it may be a drawback when unknown or mixed exposures are encountered. In these situations only classic biological monitoring methods, such as the thioether test [13,14] and the urinary mutagenicity assay [15], can be used. A less selective method for the measurement of the degree of alkylation of certain nucleophilic centres in haemoglobin would have the advantage that a risk-related parameter could be determined even when the chemical origin of this risk remains unknown.

The thiol group of the β-93 cysteine in haemoglobin is a readily accessible nucleophilic centre. It is the only reactive thiol group in haemoglobin and, therefore, seems most promising for the development of a non-selective alkylation assay. Alkylation of this thiol group leads to gross changes in the biochemical and biophysical nature of the haemoglobin. In a first attempt to use these features, alkylation of the thiol group was measured spectrophotometrically by titration with 4,4′-dithiopyridine (4-PDS) [16]. However, this method lacks the sensitivity needed for the monitoring of biological effects at the low exposure levels that occur in practice. Therefore, we developed a method with a lower detection limit using isoelectric focusing (IEF) in ultra-thin polyacrylamide gels with a non-linear pH gradient.

EXPERIMENTAL

Chemicals

Acrylamide, ammonium persulphate, bisacrylamide and riboflavin monophosphate (electrophoresis purity) were from Bio-Rad (Richmond, CA, U.S.A.), Amberlite MB-6 (20–50 mesh) was from Serva (Heidelberg, F.R.G.), diaphorase and NADH were from Boehringer (Mannheim, F.R.G.), dimethyl sulphate (DMS), 4,4′-dithiopyridine (4-PDS), iodoacetamide (IAA), methylmethanesulphonate (MMS), 3-(trimethoxysilyl)propylmethacrylate (TMSPM) and N,N,N′,N′-tetramethylethylenediamine (TEMED) were from Janssen (Beerse, Belgium), Ampholines (pH 6.0–8.0) were from LKB (Bromma, Sweden), β-alanine (99+%) and glycerol (doubly distilled) were from E. Merck (Darmstadt, F.R.G.). Sephadex G-25 (medium) was from Pharmacia (Uppsala, Sweden). All other chemicals were of analytical purity. Only freshly deionized distilled water was used.

Apparatus and glassware

All glassware was acid-washed prior to use in order to remove Cu^{2+} ions, which can catalyse the oxidation of haemoglobin even at trace concentrations [17]. The ultra-thin layer electrofocusing gel caster and photopolymerization light were from Bio-Rad. Filters (0.45 μm, type H4) were from Millipore (Bedford, MA, U.S.A.). Gels were focused on a 2117 Multiphor II focusing unit with a Macrodrive V power supply from LKB. The IEF samples were applicated with a Pratiga

D8 sample application kit from Instrumentation-Lab (IJsselstein, The Netherlands). Gels were scanned on a KS3 Microdensiscan from Kipp & Zonen/Skalar.

Isolation and purification of haemoglobin

Blood samples from healthy volunteers were collected, using EDTA to prevent coagulation. EDTA contributes as a scavenger of Cu^{2+} ions and acts as an allosteric anion modulating the oxygen binding [18]. Immediately after collection the blood samples were cooled to 4 °C to prevent carboxypeptidase activity [19]. All of the following purification steps were carried out at 4 °C. To 3 ml of blood, 6 ml of ice-cold phosphate-buffered saline, pH 8.0 (PBS; 10 m*M* sodium phosphate plus 150 m*M* sodium chloride plus 1 m*M* EDTA) were added. The samples were centrifuged at 2300 *g* for 5 min. Serum and buffy coat were removed by careful suction, and the erythrocytes were resuspended with 8 ml of PBS. After mixing, the samples were centrifuged again at 2300 *g* for 5 min. The supernatant was removed by careful suction, and a few erythrocytes were sacrificed to remove any remaining buffy coat. This washing procedure was repeated twice. The erythrocytes were then layered on a solution of 30% sucrose, containing 0.5% Triton X-100 and 10 m*M* Tris (pH 9.0), and lysed by centrifugation at 900 *g* for 10 min as described by Scott [20]. To the lower part of the sucrose layer, containing the haemoglobin, 1/10 volume of 1 *M* sodium chloride was added, to optimize the density towards the removal of cellular debris [18], and it was centrifuged once more at 110 000 *g* for 15 min. The supernatant was equilibrated by leading gaseous carbon monoxide through the solution for 5 min, to convert the haemoglobin into carboxyhaemoglobin, which is more stable against oxidation to methaemoglobin. The temperature must be adjusted to 37 °C during the equilibration. All further steps were carried out in solutions saturated with carbon monoxide at 4 °C. The supernatant was dialysed twice against at least 50 volumes of PBS for several hours. Finally the haemoglobin was centrifuged once more at 10 000 *g* for 20 min to remove any remaining cellular debris or denaturated proteins. The resulting haemoglobin solution can be stored under carbon monoxide at −18 °C when the pH is kept between 8.0 and 8.5. Protein concentrations of the resulting haemoglobin solution were determined by the Lowry method. Haemoglobin was determined by a modified haemoglobin–cyanide procedure, and the chemical determination of free sulphydryl groups was done by titration with 4-PDS, both as described by Neis et al. [16]. Immediately before application to the IEF gels the haemoglobins were desalted with Sephadex G-25.

In vitro incubation with alkylating agents

In a shaking water-bath (210 rpm), closed sterile vials each containing 3 ml of blood with EDTA as anticoagulant, or 3 ml of washed erythrocytes suspended in PBS, or 3 ml of a purified haemoglobin solution (Hb concentrations ca. 50 mg/ml in all cases) and 100 µl of a sterile solution of the compound under test were incubated at 37 °C for 5 h. DMS and MMS were dissolved in saline, IAA in dimethyl sulphoxide (DMSO).

Preparation of IEF gels

Solutions of 24.25% acrylamide (w/v), 0.75% bisacrylamide (w/v), 2% ammonium persulphate (w/v) and 25% glycerol (w/v) were stored at −18°C. A solution of 0.1% (w/v) riboflavin 5′-monophosphate was protected against light and stored at 4°C. All stock solutions were stored in small portions for single usage, under nitrogen.

To a mixture of acrylamide, bisacrylamide and glycerol solutions (4 ml each) and 8 ml of β-alanine solution (62.5 mg/ml), 500 mg Amberlite MB-6 were added for further purification. The mixture was stirred for 1 h, and the Amberlite was removed by filtration through 0.45-μm filters. Then 1.25 ml of Ampholines, 125 μl of riboflavin 5′-monophosphate and 150 μl of ammonium persulphate solution were added, and the mixture was deaereated (in the dark to prevent polymerization). Ultra-thin T5/C3 gels of 0.4 mm thickness and 10 cm length were cast immediately after the addition of 10 μl of TEMED, using glass plates coated with TMSPM as matrix. The gels were allowed to photopolymerize for 8–12 h. Gels could be stored in sealed packages with water-saturated nitrogen at 4°C.

Procedure for IEF

The gels were applied to the focusing unit, which was thermostated at 10°C. The anode solution was 0.25 *M* HEPES and the cathode solution 0.1 *M* sodium hydroxide. Gels were prefocused for 15 min at a constant power of 10 W. After the samples were applied at the anodic side, the gels were focused for 1 h at a constant power of 10 W with a voltage cut-off at 2000 V, which was reached after ca. 20 min. For the last 10 min the voltage was adjusted to 2500 V.

Fixation and staining

After focusing, the gels were fixed for 30 min in a solution of 4% (w/v) sulphosalicylic acid, 12% (w/v) trichloroacetic acid and 30% (v/v) methanol. The gels were stained with bromophenol blue and destained with acidic alcohol as described by Awdeh [21].

RESULTS

The haemoglobin solutions were ca. 85% pure when expressed on a haemoglobin/protein basis. When haemolysis was performed by addition of water, instead of with the sucrose procedure, only 80% purity was obtained. Although the methaemoglobin form and a partly oxidized intermediate of the carbon monoxide-saturated HbA could easily be distinguished on the gels when haemoglobin was stored under air, only small amounts of these oxidized haemoglobins were present when the procedure described above was followed precisely. DMSO slightly stimulated the formation of oxidized haemoglobins.

Several minor haemoglobins, including HbA_{1c}, HbA_2 and HbF, were visible as distinct bands. The glutathione adduct HbSSG and a heparin adduct were also visible when their formation was induced by addition of glutathione and heparin. The detectability of the heparin adduct under those circumstances, together with

Fig. 1. Example of an IEF gel with haemoglobins alkylated by iodoacetamide. Lane 1 was treated only with DMSO; in lane 2 moderate and in lane 3 high degrees of alkylated haemoglobin are visible.

the high affinity of heparin towards haemoglobin [22], supported the choice of EDTA in stead of heparin as anticoagulant.

After IEF of haemoglobin treated with alkylating agents, extra bands became visible slightly to the anodic side of the normal haemoglobins. In IEF gels with a linear pH 6–8 gradient the alkylated HbA was positioned at ca. 1 mm to the anodic side from normal HbA, but it was not completely separated from HbA_{1c}. When gels with a non-linear pH gradient were used the distance to normal HbA was enlarged to ca. 4 mm. The position of HbA_{1c} in these gels was at ca. 2 mm to the anodic side from the alkylated product (Fig. 1). There was no difference in p*I* between the product formed after alkylation of haemoglobin with IAA and the product formed after reaction with the methylating agents DMS and MMS.

The formation of an alkylated product from human haemoglobin, after in vitro treatment with IAA, MMS and DMS, was determined by densitoscan. The results are illustrated in Fig. 2.

The reaction of IAA with purified haemoglobin was very effective. All HbA was alkylated at an IAA concentration as low as 1 m*M*. With erythrocytes and whole blood the alkylation is much less effective. At an IAA concentration of 1 m*M* only 10% of the HbA became alkylated. These results were in good correspondence with those obtained with the 4-PDS method.

Methylation of HbA groups by DMS and MMS is less effective than alkylation by IAA. Again the alkylation in erythrocytes and whole blood is less effective

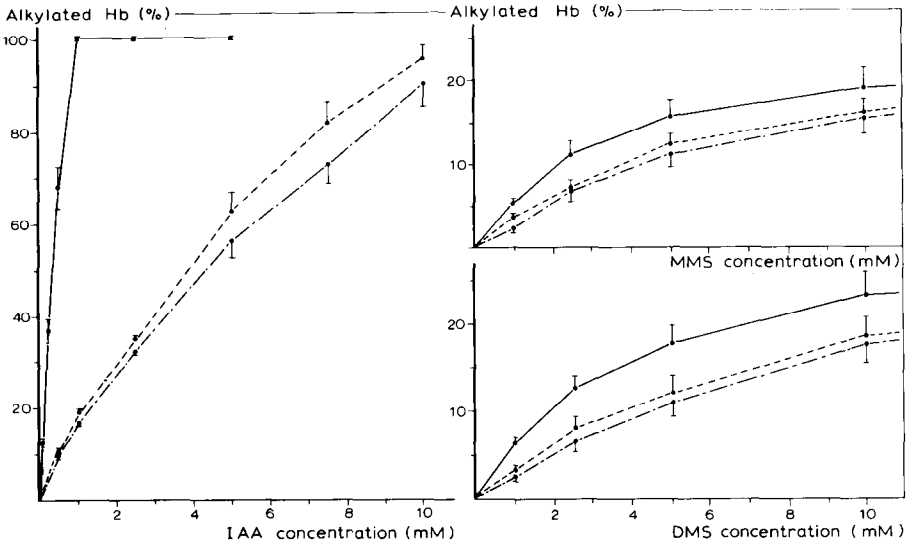

Fig. 2. Amount of alkylated human haemoglobin formed after treatment of whole blood (——), washed erythrocytes (-----) and purified haemoglobin (— · — · —) with iodoacetamide, methylmethanesulphonate and dimethyl sulphate determined with IEF. Mean and standard deviation represent six determinations.

than with purified haemoglobin, but the difference is much less pronounced than in the case of IAA. The values for the methylated product determined by the IEF–densitoscan method were in good agreement with those for the disappearance of free sulphydryl group determined with the 4-PDS method, provided the haemoglobins were converted into their oxy form with diaphorase prior to titration with 4-PDS [22]. Without this pecaution the thiol methylation values determined by the 4-PDS method were much lower, owing to formation of methaemoglobin. At higher MMS and DMS concentrations a smear of unknown haemoglobin adducts became visible in the gels.

In haemoglobin samples not treated with alkylating agents a minor amount of an unknown protein could be detected in the exact position of the alkylated haemoglobins. The amount of this protein was $0.45 \pm 0.26\%$ ($n = 18$).

DISCUSSION

The use of IEF in polyacrylamide gels for the determination of minor haemoglobins has been suggested by several authors [22,23]. When precautions are taken to keep the haemoglobins in their native state, very small amounts of haemoglobins that differ only slightly from the main form can be detected. The resolution can be increased further when non-linear pH gradients, which have a much shallower slope around pH 7, are used [24].

In this study a product formed after in vitro alkylation of haemoglobin was determined. The sulphydryl group of cysteine is one of the most reactive nucleophilic centres in proteins. Since there is only one such a reactive sulphydryl group,

at the β-93 cysteine, in each $\alpha\beta$ haemoglobin dimer, the distinct alkylated haemoglobin was expected to be HbA alkylated at this β-93 cysteine. This expectation was confirmed by the good correspondence between the amount of alkylated haemoglobin determined by densitoscan of the IEF gels and the disappearance of free sulphydryl groups determined by titration with 4-PDS. Furthermore, with IAA, which is known to be a selective thiol reagent, a considerably higher amount of alkylated product was formed at the same concentrations than with the less selective methylating agents MMS and DMS. This low selectivity is also expressed in the smear of other adducts seen in the IEF gels at higher concentrations of MMS and DMS.

The alkylation of purified haemoglobin was higher than the alkylation of haemoglobin in erythrocytes for all three compounds. Both the impermeability of the erythrocyte membrane and interferences with intracellular compounds may contribute to this feature. The remarkably high difference for IAA, in comparison with the smaller differences found for MMS and DMS, is probably due to competing reactions with other thiols, such as the intracellular glutathione. Another explanation may be found in a lower permeability of the erythrocyte membrane for IAA.

It is interesting to note the small difference between the extent of alkylation in washed erythrocytes and in whole blood for all three compounds. This shows that detoxification and competing reactions in the plasma are of little importance.

In haemoglobin not treated with alkylating agents, a minor protein band was detected with the same p*I* as alkylated HbA. At low degrees of alkylation this protein could not be separated from the alkylated HbA. The amount of this protein was between 0.2 and 0.7% of normal HbA. It is possible that this minor protein band represents a background of alkylated HbA present in human blood. Bailey et al. [4] reported a background of ca. 0.02% methylated cysteine in human haemoglobin as determined by gas chromatography–mass spectrometry. Since only one out of three cysteines present in HbA has a high chemical reactivity it is probable that the background methylation of this β-93 cysteine is ca. 0.06%. When the expected background alkylations other than methylation are taken into account, total background alkylations of the β-93 cysteine between 0.2 and 0.7% are not unlikely.

The method as developed can be of use in further studies on haemoglobin alkylation in vitro. More important is its possible use for the monitoring of biological effects. In accordance with the main features of the method, namely the unimportance of the exact nature of the alkylating agent and the relatively high background in unexposed persons, the most important application will probably be found in studies on a group basis in circumstances with mixed exposures (e.g. waste incinerators).

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