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Mannitol Prevents Methionine Sulphoxidation Mediated Electrophoretic Heterogeneity of Apolipoprotein A-I

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Hybrid isoelectic focusing of apolipoprotein A-I in polyacrylamide gels with immobilized pH-gradients under non-denaturing conditions resulted in the occurrence of additional bands which could prevent the specific and sensitive detection of genetic variants. Hybrid isoelectric focusing of two chromatographically distinguishable apolipoprotein A-I isoforms that differ by sulphoxidaton of methionine residues, apo A-I(Met) and apo A-I(MetSO), revealed that the additional bands were caused by this post-translational modification. Several antioxidative additives and conditions were compared for their ability to prevent methionine sulphoxidation in apolipoprotein A-I. In the presence of 200 g/L mannitol in the gel, apolipoprotein A/I focused as a single band. Since methionine sulphoxidation in proteins is a general phenomenon either taking place in vivo or in vitro by isoelectric focusing, we conclude that isoelectric focusing in the presence of mannitol will improve the quality of resolution of many proteins in gels with immobilized pH-gradients.

INTRODUCTION

Isoelectric focusing (IEF) of proteins under denaturing conditions helps to detect genetic variants in which amino acid changes lead to complete net charge changes, but mostly fails to detect variants in which amino acid substitutions lead to only slight net charge changes. Previously, hybrid isoelectric focusing (HIEF) in gels with immobilized pH-gradients (IPG) (Atland and Rossmann, 1985; Fawcett and Chrambach, 1985; Rimpilainen and Righetti, 1985) helped to identify some genetic variants which resulted from electrically neutral amino acid substitutions (Atland *et al.*, 1986, 1987; von Eckardstein *et al.*, 1990). The sensitivity of this technique towards the detection of genetic variants with electrically neutral amino acid substitutions was improved by performing IEF at conditions that preserve an intermediate state between denaturation and renatureation, since denaturation shifts the isoelectric point (IP) of many proteins towards more basic pH-values (Ui, 1971, 1973; Salaman and Williamson, 1973; Hobart, 1975; Altland *et al.*, 1981, 1982; Hackler *et al.*, 1985). Theoretically, some amino acid substitutions should change this labil, intermediate state either towards renaturation or towards denaturation and thus lead to a shift in the IP of the protein to the anode or cathode, respectively. It has been demonstrated by Ramshaw *et al.* that some 85% of electrically neutral substitutions can be detected by electrophoresis under conditions keeping the test protein in a near native state (Ramshaw *et al.*, 1979). However, conformational stability of proteins at borderline conditions is influenced not only be genetic but also by post-translational variations, some of which are produced by IEF itself. For example, it has been suggested that IEF favours the oxidation of cysteine and methonine residues, resulting in the appearance of cysteic acid in the former or methionine sulphoxide in the latter case. These structural changes may in turn result in the appearance of spurious bands thereby preventing the detection of naturally occuring variants.

The occurrence of unspecific anti-apo A-I immunoreactive bands was a major drawback in our trials to establish an electrophoretic screening procedure towards the detection of electrically neutral mutants of apo A-I by the use of HIEF under non-denaturing conditions. Since sulphoxidation of methionine residues in apo A-I causes a chromatographic polymorphism and possibly also conformational changes (Anantharamaiah *et al.*, 1988; von Eckardstein *et al.*, 1990) and since IEF has been demonstrated to sulphoxidize methionine residues in general (Jacobs 1973), we tested whether the addition of various antioxidative substances to the gel prevents the formation of the additional unspecific bands.

EXPERIMENTAL

Sample preparation. Two volumes of EDTA plasma were mixed with one volume of a buffer containing 10 g/L sodium-dodecylsulphate and 200 g/L glycerol and incubated for 2 h at 37°C (Atland *et al.*, 1980). In some experiments, plasma proteins were oxidized by the incubation of 100 µL plasma with increasing amounts of chloramine T (Sigma, St. Louis, MO, USA) for 3 h at room temperature (von Eckardstein *et al.*, 1991).

Demonstration of apo A-I by hybrid-isoelectric focusing in immobilized pH-gradients. Apo A-I was demonstrated by double-one dimensional electrophoresis in the sequence: PAGE → HIEF in polyacrylamide gels with IPG, principally

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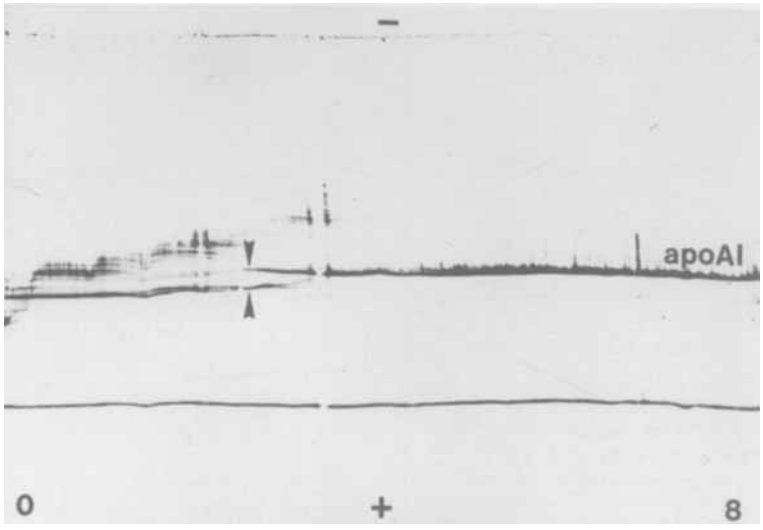

Figure 1. Effect of urea on HIEF of A-I. HIEF was was performed in a gel with an immobilized linear pH-gradient pH 4–7 rehydrated with 7.5 g/L carrier ampholytes, 50 mM dithiotreitol, 2 g/L PEG and a concentration gradient of urea perpendicular to the pH-axis which ranged from 0 to 8 M. HIEF was performed for 6 h at 5000 V and a maximum of 5 mA. Note the intermediate state of apo A-I around pH 3 (indicated by arrows).

as described by Atland and coworkers (Atland *et al.*, 1980) but introducing some modifications for the second dimension. Briefly, vertical PAGE was performed in slab gels (T = 5%, C = 3%) using the DESAGA VA sytem (Heidelberg, Germany), a cathodic buffer with 0.04 mol/L Tris, 0.23 mol/L glycine (pH 8.9), and bromphenol blue and an anodic buffer with 0.12 mol/L Tris-chloride (pH 8.08). After a pre-run, in which the bromphenol blue front migrated 4 cm into the gel, 20 µL pretreated samples (= 6.6 µL plasma) were applied to the sample wells and electrophoresis was continued until the bromphenol blue front has migrated 2 cm more. A 10 mm wide gel strip containing the apo A-I of several adjacent samples was removed from the gel and transferred to another gel for HIEF. HIEF was performed in polyacrylamide gels (T = 5%, C = 3%), with a nonlinear IPG containing a flattened part with a maximal pH slope of 0.04 pH-units/cm with the IP of apo A-I in its centre as previously described by Atland (Atland and Atland, 1984). The washed and dried gels were rehydrated with a solution containing 3 and 8 M urea for non-denaturing and denaturing conditions, respectively, 7.5 g/L carrier ampholytes pH 4–6 and pH 5–7 (Pharmacia) at a ratio 2:1, 50 mM dithiotreitol and polyethylene glycol 6000 (PEG). This solution was varied as indicated in the results section by the addition of the following agents to prevent oxidation: ethylendiamintetraacetate (EDTA); ascorbic acid; desferyoxamine mesylate; or mannitol (Sigma, St. Louis, MO, USA). In some indicated experiments HIEF was performed in gels with IPG which were rehydrated with a concentration gradient of additives (urea, mannitol) perpendicularly to the pH-axis. HIEF was performed fo 16 h with 4000 V and 5 mA. The gel was then stained with Coomassie Blue.

Demonstration of the chromatograhic heterogeneity of apo A-I and isolation of reduced and methionine sulphoxidized apo A-I. Reduced and methionine sulphoxidized isoforms of apo A-I were demonstrated and isolated by reversed phase HPLC as previously described by our laboratory (von Eckardstein *et al.*, 1991). Briefly, 100 µg of undelipidated HDL apoproteins were separated by reverse phase HPLC using C 18 widepore column (300 A, Baker, Philipsburg, NJ, USA). The gradient was started with 40% acetonitrile and linearly increased to 90% within 50 min. The oxidized and non-oxidized isoforms were collected, liophilized and stored at −70°C.

RESULTS

The effect of antioxidatives on HIEF of apo A-I. Figure 1 demonstrates the separation of apo A-I by HIEF in a gel which was rehydrated perpendicualrly to the pH axis with an urea gradient ranging from 0 to 8 M. At urea concentrations exceeding 3 M, the isoelectric point of apo A-I was changed towards a more basic pH indicating complete denaturation of apo A-I. Around 3 M urea apo A-I preserves an intermediate unstable state between denaturation and renaturation. In this state even slight post-translational modifications may alter the conformation and thereby the IP of apo A-I. Actually HIEF of apo A-I in a gel containing a flattened IPG and rehydrated with 3 M urea resulted in the occurrence of several apo A-I immunoreactive bands, which would prevent the detection of electrically neutral apo A-I variants. We hypothesised that the encountered spurious bands reflect post-translational modification of apo A-I, e.g. by oxidation. We therefore tested the antioxidative effect of varous additives on HIEF of apo A-I in 3 M urea. Addition of ascorbic acid, EDTA, or desferoxyamine mesylate to the rehydration solution, as well as of HIEF in paraffin oil protected gels were ineffective to prevent the occurrence of additional bands (*not shown*). By contrast, rehydration of the gel in the presence of mannitol led to the demonstration of apo A-I as a single band also under non-denaturing conditions. In the gel rehydrated with 3 M urea containing a mannitol concentration gradient ranging from 0 to 200 g/L perpendicular to the pH-axis, the IP of apo A-I was shifted by approx. 0.05 pH units towards anode (Fig. 2). The shift occurred at a mannitol concentration of 50 g/L and points to the protective effect of this compound on the structure of apo A-I.

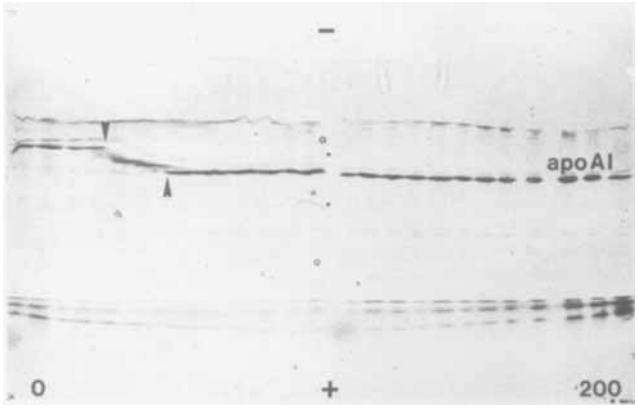

Figure 2. Effect of mannitol on HIEF of apo A-I under non-denaturing conditions. HIEF was performed in a gel with a nonlinear immobilized pH-gradient p-H 4–7, which was rehydrated with 3 M urea, 7.5 g/L carrier ampholytes, 50 mM dithiothreitol, 2 g/L PEG and a concentration gradient of mannitol perpendicular to the pH-axis which ranged from 0 to 200 g/L. HIEF was performed for 6 h at 5000 V and 5 mA. Note the shift in the IP of apo A-I to a lower pH at approximately 30 g/L mannitol (indicated by arrows).

Effect of mannitol on HIEF of methionine sulphoxidized apo A-I. Previously, our and another laboratory demonstrated that sulphoxidation of methionine residues 112 and 148 in apo A-I causes the chromatographic heterogeneity of apo A-I (15, 16) (Fig. 3). To test whether methionine sulphoxidation in apo A-I is responsible for the occurrence of additional bands which were prevented by the addition of mannitol, apo A-I(Met) and apo A-I(MetSO) were separated by reversed-phase HPLC and subsequently electrophoresed by HIEF in gels with 3 M urea in the presence or absence of mannitol (Fig. 3). In the presence of mannitol, apo A-I(Met) focused as a single band. Apo A-I(MetSO) occurred as two isoforms, the minority at the IP of apo A-I(Met), the majority at a more cathodic IP. In the absence of mannitol, however both apo A-I(Met) and apo A-I(MetSO) exhibited the IP of apo A-I(MetSO).

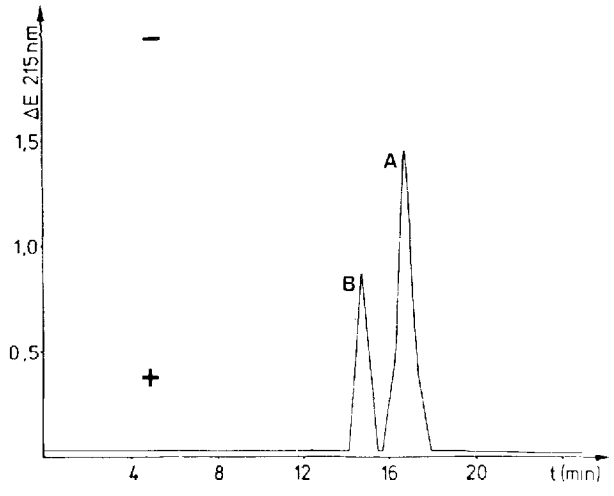

Figure 3. Demonstration of reduced and methionine sulphoxidized apo A-I by reversed phase HPLC and HIEF under nondenaturing condiitons. Fraction and lanes A correspond to the reduced apo A-I isoform apo A-I(Met), fraction B and lanes B the oxidized isoform apo A-I(MetSO). (w) denotes HIEF of apo A-I in the presence of mannitol, (w/o) denotes HIEF in the absence of mannitol. Note that apo A-I(Met) and apo A-I(MetSO) preserve distinct isoelectric points in the presence of mannitol whereas in the absence of mannitol, apo A-I(Met) and apo A-I(MetSO) exhibit identical IP.

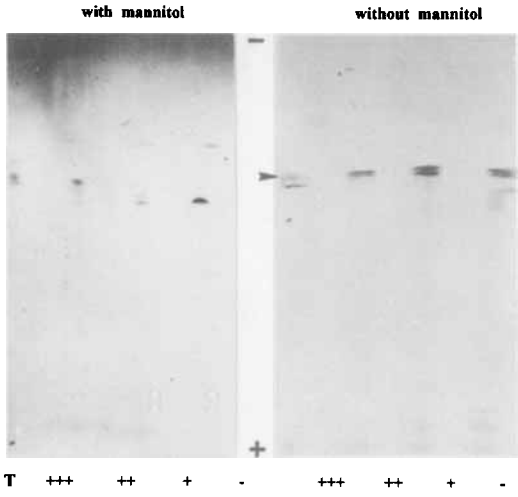

Figure 4. Effect of mannitol on HIEF of apo A-I oxidized with chloramine T. Plasma was incubated with increasing amounts of chloramine T for 16 h at 37°C. The samples were then separated by HIEF in the presence (left) or absence (right) of 200 g/L mannitol in the gel. In the absence of mannitol, apo A-I oxidized with chloramine T focuses as a single band with the IP of apo A-I(MetSO) (arrow) whereas in the presence of mannitol apo A-I occurs as several bands with IP of apo A-I(Met) and apo A-I(MetSO) (arrows).

These results suggested that mannitol prevents methionine sulphoxidation during IEF. To strengthen this hypothesis, plasma was incubated with increasing concentrations of chloramine T, which facilitates methionine sulphoxidation in apo A-I, and subsequently separated by HIEF either in the absence or in the presence of mannitol (Fig. 4). In the absence of mannitol, apo A-I focused at the isoelectric point of apo A-I(MetSO). By contrast, in the presence of mannitol, apo A-I was separated into several bands. Their number and IP increased with the concentration of chloramine T. This indicates that mannitol pevents the oxidation of apo A-I during IEF and further supports our hypothesis that the electophoretic heterogeneity of apo A-I under non-denaturing conditions is at least partially due to methionine sulphoxidation in apo A-I.

DISCUSSION

Only a proportion of amino acid substitutions in proteins leads to net charge changes which are sufficient to detect these genetic variants by the use of IEF. Some electrically neutral amino acid substitutions affect the conformational stability of proteins. This facilitates denaturation and thereby changes their IP (Atland *et al.*, 1981, 1982). Theoretically, therefore, IEF of proteins at borderline conditions, at which they preserve a state between renaturation and denaturation, should serve as a valuable tool for the detection of genetic variants with electically neutral amino acid substitutions. Actually, several otherwise undetectable variants of transthyretin could be separated from the respective normal allele products following to this concept (Altland *et al.* 1987). However, also post-translational modifications such as oxidation, which occurs during IEF, may affect the structural stability and thereby IP of proteins. This in turn may give rise to the occurrence of unspecific additional bands. In our studies, this

problem occurred whilst we searched for a method to identify electrically neutral variants of apo A-I. Previously, we have demonstrated that oxidation of methoinine residues 112 and 148 to the corresponding sulphoxides in apo A-I may exert local effects on its structure and stability (Jonas *et al.*, 1993) and thereby contribute to the distinct physicochemical properties of oxidized and reduced isoforms. For example, the retention time of apo A-I(Met) upon reversed phase HPLC is shorter as compared to apo A-I(Met) (Fig. 3) indicating the lower hydrophobicity of apo A-I(MetSO). In the present study we found that under non denaturing conditions apo A-I(Met) and apo A-I(MetSO) differ in their isoelectric points by approximately 0.05 pI-units. This observation points to the possible role of methionine sulphoxidation for the occurrence of the unspecific anti-apo AI immunoreactive bands. Methionine sulphoxidation may occur during IEF (Jacobs, 1973). To eliminate contact between the gel and oxygen of the atmosphere, IEF was initially performed in gels that were covered with paraffin oil as described by Atland *et al.* (Atland *et al.*, 1987). This procedure did not prevent the occurrence of unspecific bands. Hence, the oxidizing activity had to bae ascribed to the gel matrix rather than to oxygen in the ambient atmosphere. One explanation for the oxidizing activity in the gel was the resence of transition metal ions which favour the formation of free oxygen radicals which in turn are potent oxidants. For their elimination, we tested the addition of chelators to the gel. Neither EDTA nor desferyoxyamine mesylate prevented the occurrence of spurious bands.

Recently, Cossu and colleagues (Cossu *et al.*, 1990) suggested that IEF in immobilized pH-gradients would oxidize sulphur containing amino acids in proteins through amine oxides ($R_3N^+O^-$) or nitrosoamines which are produced in immobilines during polymerization by ammonium persulphate. Short washing of the gels in the presence of ascorbic acid, as suggested by these authors, was no remedy in our hands, since ascorbic severely increased the conductivity of the gel and thereby unacceptably prolonged the time of electrophoresis. Moreover, ascorbic acid was suggested by Cossu *et al.* to prevent oxidation of cysteine residues which, however, are not present in apo A-I. Neither the addition of dithiotreitol to the rehydration solution (Hackler *et al.*, 1985) prevented the occurrence of the contaminating bands.

Finally, IEF in the presence of mannitol, an uncharged molecule of well known antioxidative properties, helped to demonstrate apo A-I as a single band exhibiting the IP of the non-denatured protein. In the presence of mannitol, apo A-I(Met) isolated by HPLC preserved its IP anodic from apo A-I(MetSO). In the absence of mannitol, both isoforms focused at the cathodic IP of apo A-I(MetSO) (Fig. 3). We, therefore, conclude that mannitol prevents methionine sulphoxidation in apo A-I during IEF, and thereby changes of the IP of the protein. The mechanism by which mannitol prevents methionine sulphoxidation remains unclear. One possibility is that mannitol directly prevents the nucleophilic attack of N-oxides. Another possibility is based on the assumption that sulphur centred radicals produced during the polymerization of acrylamide and eventually trapped in the gel are effectively scavenged by mannitol.

In conclusion, oxidation of methionine residues in apo A-I during IEF appears to cause electrophoretic heterogeneity which corresponds to the previously described chromatographic one and which is suppressed by addition of mannitol to the gel. Since methionine sulphoxides are present in many native proteins and, moreover, since IEF generally favours methionine sulphoxidation (Brot and Weissbach, 1983; Swaim and Pizzo, 1988), it is conceivable that addition of mannitol to polyacrylamide gels will help to improve IEF of many other proteins, especially when IEF is performed in immobilized pH-gradients under non-denaturing conditions.

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