EFFECT OF DIAMIDE ON OLIGOMERIZATION OF WATER-SOLUBLE RABBIT LENS PROTEINS

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The development of senile cataract in man is accompanied by covalent polymerization of proteins and a decrease in the content of sulfhydryl groups in the composition of the crystallins of the lens [1, 4, 8-10]. It was shown previously that incubation of lenses with diamide (azodicarboxylic acid *bis*-dimethylamide), which specifically oxidizes SH-groups to disulfides, leads to the development of opacity of the lens [6]. The mechanism of development of this diamide-induced opacity has not yet been established. The possibility cannot be ruled out that the action of diamide simulates the formation of senile cataract through oxidation of SH-groups of the crystalline, the principal proteins of the lens, followed by their oligomerization through a S=S bond, leading to a decrease in their solubility.

The aim of this investigation was to study the ability of diamide to induce oxidation of SH-groups of rabbit lens crystallins and to determine whether the formation of protein oligomers is the result of oxidation of SH-groups.

EXPERIMENTAL METHOD

Rabbit lenses were frozen at −25°C and kept in an atmosphere of argon before use. All procedures (homogenization of the tissue, centrifugation, and dialysis) were carried out at 5°C in an atmosphere of argon. The lens was homogenized in 1 ml of medium containing 100 mM NaCl, 5 mM sodium azide, 0.1 mM EDTA, 10^{-5} M phenylmethylsulfonyl fluoride, and 10 mM HEPES, pH 7.2, at 5°C (medium A), and allowed to stand for 1 h for extraction. The insoluble material was removed by centrifugation at 40,000*g* at 30 min. The supernatant was dialyzed for 12 h at 5°C in medium A. The dialyzed extract of water-soluble proteins of the lens was incubated with diamide for 1 h at 37°C in medium A in an atmosphere of argon. After incubation, sodium dodecylsulfate (SDS) was added to the extract of proteins in the ratio of 1:1 by weight with protein. Extract of water-soluble proteins (3-5 mg protein) was applied to a column (1.6 × 41 cm), packed with Ultrogel AcA 44 and eluted with medium A containing 0.1% SDS at the rate of 15 ml/h at 20°C. Chromatography was carried out in an atmosphere of argon. Elution of the protein concentration was determined by the biuret reaction in the presence of 1% Na deoxycholate. Bovine serum albumin was used as the standard. SH-groups in the extract of water-soluble proteins were determined with the aid of 5.5′-dithio-*bis*-(2-nitrobenzoic acid) (DTNB) [5] in medium containing 0.5 mM DTNB, 100 mM NaCl, 5 mM NaN_3, 0.1 mM EDTA, and 50 mM Tris-HCl (pH 8.3, at 37°C) in a thermostated quartz cell with a capacity of 3 ml, with mixing. The reaction was initiated by the addition of 3 μl of protein extract. To convert changes in optical density of the incubation medium at 412 nm into the number of SH-groups a calibration curve obtained when determining interaction between DTNB and reduced glutathione was used. Electrophoresis in 15% polyacrylamide gel was carried out in the presence of SDS by Laemmli's method [7].

Determination of SH-groups in extract of water-soluble lens proteins with the aid of DTNB showed that the extract contained 64 ± 0.5 mmoles SH-groups per milligram protein. All the SH-groups determined in the extract belonged to lens proteins, for after precipitation of the proteins by the addition of 5% TCA to the extract, no SH-groups could be found in the

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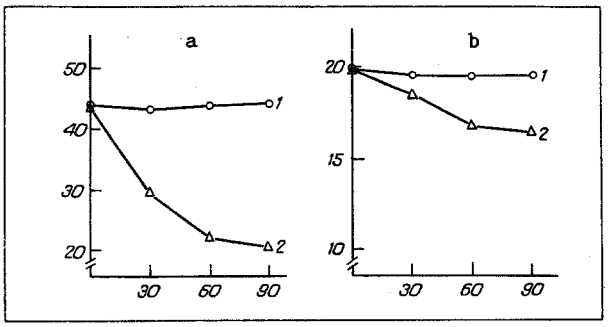

Fig. 1. Action of diamide on content of fast (a) and slow (b) SH-groups in extract of water-soluble proteins of rat lens. Incubation in the absence (1) or in the presence (2) of 2 mM diamide. Ordinate, content of SH-groups (in mmoles/mg protein). Abscissa, time (in min).

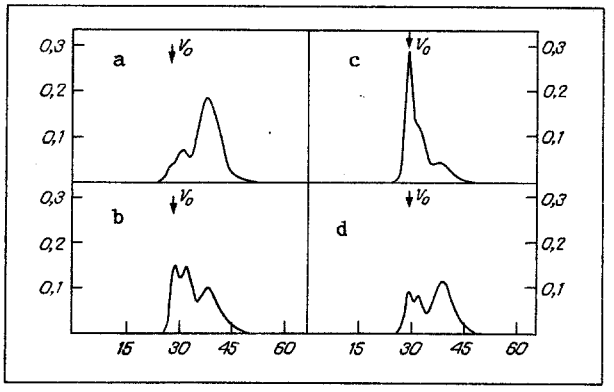

Fig. 2. Gel-filtration of extract of water-soluble rabbit lens proteins. Specimen incubated for 1 h in absence (a) or in presence of 1 mM (b) or 2 mM (c, d) of diamide. In d) after incubation with 2 mM diamide for 1 h, the extract was incubated for 30 min at 37°C in an atmosphere of argon and in the presence of 10 mM glutathione. Ordinate, optical density at 280 nm. Abscissa, volume (in ml).

extract. The absence of nonprotein SH-groups in the extract is the result of preliminary dialysis, for in the undialyzed extract of water-soluble lens proteins about 20% of their total content of SH-groups was nonprotein in nature, and evidently belonged to glutathione, a concentration of which in the lens is 1-10 mM [3].

SH-groups of lens proteins interacting with DTNB were divided into two groups: fast, interacting with DTNB in the course of 30 sec, and slow, the reaction with which occurred in the next 4 min. The quantity of fast SH-groups was about 44 mmoles/mg protein and that of slow SH-groups about 20 mmoles/mg protein. Oxidation of fast and slow SH-groups under the influence of diamide was investigated. Incubation of the extract of water-soluble lens proteins with diamide for 1.5 h at 37°C in an atmosphere of argon led to a gradual fall in the content of fast and slow SH-groups. Incubation of the extract under these same conditions in the absence of diamide did not affect the content of SH-groups. The effect of diamide depended on the incubation time. With diamide in a concentration of 2 mM, in the course of 1 h of incubation 45-50% of the fast SH-groups were oxidized, but only 15% of slow SH-groups (Fig. 1). It can be tentatively suggested that diamide oxidizes mainly peripheral SH-groups, which are easily accessible for DTNB.

To investigate covalent oligomerization of water-soluble lens proteins the method of gel-filtration was used. Ultrogel AcA 44, which has an exclusion limit at about 200 kD, was used as the chromatographic carrier. To prevent oligomerization of the proteins on account of noncovalent interactions, chromatography was performed in the presence of 0.1% SDS. Uncontrolled oxidation of SH-groups during chromatography was ruled out because the chromatography was carried out in an atmosphere of

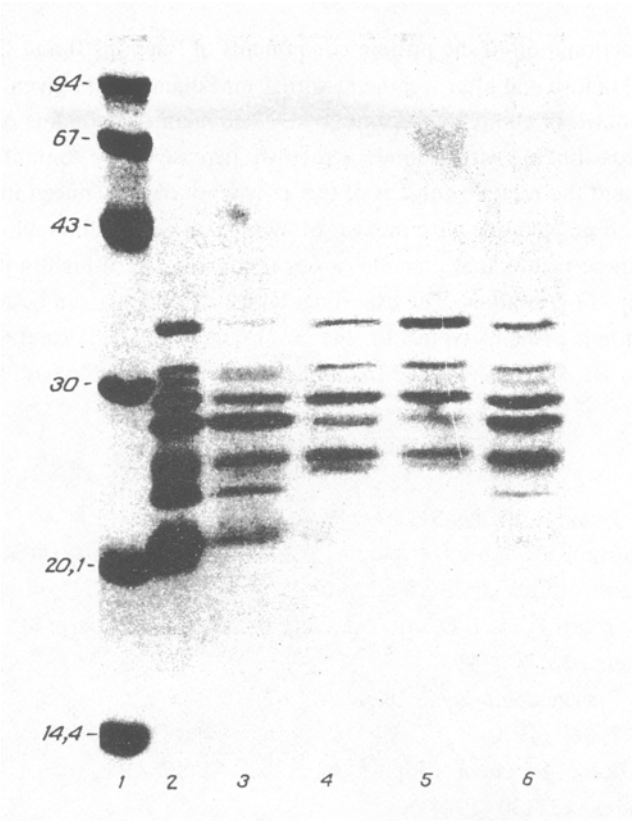

Fig. 3. Polyacrylamide gel electrophoresis. 1) Standard set of proteins: phosphorylase B (94 kD), bovine serum albumin (67 kD), egg albumin (43 kD), carboanhydrase (30 kD), soy trypsin inhibitor (20.1 kD), and α-lactalbumin (14.4 kD); 2) rabbit lens proteins; 3-6) fractions II (29-34 ml; 4, 5) and III (35-40 ml); 3, 6) obtained after gel-filtration of extract of soluble lens proteins; 3, 4) after incubation with 1 mM diamide; 5, 6) control.

argon. The elution profile of water-soluble lens proteins during their fractionation on a column with Ultrogel AcA 44 in the presence of SDS is shown in Fig. 2. Most of the protein was eluted from the column in a broad peak in the elution volume corresponding to K_{av} = 0.165 (maximum of the peak). It follows from the calibration curve, plotted from the data of gel-filtration of four proteins, namely bovine serum albumin (67 kD), egg albumin (45 kD), myoglobin (17.8 kD), and cytochrome C (12.3 kD), that this value of KAV signifies that most of the water-soluble lens proteins in the presence of SDS have a mean molecular weight of about 27 kD. It can be tentatively suggested that under the conditions of gel filtration used, the α- and β-crystallins, accounting for the greater part of the total water-soluble proteins of the rabbit lens [2], disaggregate to monomers. This enables the method of gel-filtration in the presence of SDS to be used to study the formation of oligomers of peptides of the crystallins during their covalent cross-linking, as a result of oxidation of SH-groups through the action of diamide.

It was found that incubation of the dialyzed extract of water-soluble lens proteins in the presence of diamide (0.15-2 mM) led to a decrease in magnitude of the main peak of polypeptides with an average molecular weight of about 27 kD and to the appearance of protein components with higher molecular weight, some of which were eluted in the free volume of the column. The maximal effect was observed with diamide in a concentration of 2 mM. The elution profile of water-soluble lens proteins after incubation with 1 mM and 2 mM diamide is shown in Fig. 2b, and c, respectively. It is important to note that incubation of the extract of water-soluble lens proteins in an atmosphere of argon in the absence of diamide does not lead to any appreciable oligomerization of polypeptides. Oligomerization of polypeptides under the influence of diamide is the result of the formation of S=S between monomers. This is shown by the reversibility of diamide-induced oligomerization, during the action of reduced glutathione. It will be clear from Fig. 2d that incubation of the extract of lens proteins, treated with 2 mM diamide, for 30 min with 10 mM glutathione, largely reversed the cross-linking effect of diamide.

Data on electrophoretic fractionation of the protein components of fractions II and III, obtained by gel-filtration, in the presence of SDS and dithiothreitol, before and after treatment with 1 mM diamide, are given in Fig. 3. The main components of fraction II are polypeptides with mol.wt. of about 35, 29, and 28 kD. The main components of fraction III are polypeptides with mol.wt. of 29, 28, and 26 kD. On cross-linking with diamide, a relative increase in the content of the polypeptide with mol.wt. of 29 kD was observed in fraction II, and the relative content of this polypeptide was reduced in fraction III. On the basis of these findings it can be postulated that the polypeptide with mol.wt. of 29 kD was most vulnerable to cross-linking by diamide.

It can be concluded from these results that diamide causes the formation of high-molecular-weight protein components as a result of oxidation of SH-groups of crystallins. The experimental use of diamide can be recommended for simulation of the oxidative changes brought about in lens proteins typical for the development of senile cataract.

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CHROMATOFOCUSING OF LOW-DENSITY PLASMA LIPOPROTEINS

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The heterogeneity of low-density plasma lipoproteins (LDPP) with respect to their charge [15] may be the result of the different protein composition of the lipoprotein particles of this class [10], heterogeneity of apoB — the basic protein of LDPP relative to the isoelectric point pI [11], conformational changes in apoB [13] and also, evidently, its modifications (glycosylation, damage by lipid peroxidation products [8]).

The aim of this investigation was to test chromatofocusing as a method of separating LDPP at the effective isoelectric point as a simpler method than isoelectric focusing, isotachophoresis, or ion-exchange chromatography.

EXPERIMENTAL METHOD

LDPP were isolated by single ultracentrifugation in a stepwise density gradient of NaBr [4]. Plasma was obtained from clinically healthy individuals, patients with coronary heart disease (CHD), documented by coronary arteriography, and patients

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