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# IN VITRO MODIFICATION OF BOVINE LENS ALDOSE REDUCTASE ACTIVITY

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Bovine lens aldose reductase can be activated in crude extracts upon incubation at 37°C at relatively high ionic strength. This phenomenon shows a seasonal occurrence, the enzyme being susceptible to activation only in lenses of animals sacrified in summer. Systems generating oxygen activated species induce the enzyme activation, whereas scavengers of "oxygen radicals" preserve the activated state of the enzyme. Glutathione and other thiol compounds appear to prevent the enzyme activation. 

The implication of aldose reductase in the formation of sugar cataract and peripheral neuropathies (1-3) has been widely investigated. This enzyme, the promoter of the polyol pathway, may in fact lead to sorbitol accumulation in hyperglycemia conditions, that could be detrimental for the cell (3). Several inhibitors of aldose reductase activity have been intensively studied and proposed as therapeutic tools in diabetic complications (3-6).

Bovine lens aldose reductase has been purified to homogeneity by several authors and characterized for both physical and kinetic properties (7-11). Non-linear kinetics of the homogeneous enzyme preparation, exhibiting concave downward curvatures in double reciprocal plots with respect to both NADPH and glyceraldehyde concentrations (8,9,11) have been ascribed for this monomeric protein either to an allosteric-type behaviour (8) or to an effect on the enzyme activity measurement caused by the formation of the autoxidation products of glyceraldehyde (9).

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In this paper we report evidence for in vitro enhancement of bovine lens aldose reductase activity upon incubation of the lens extract at relatively high ionic strength conditions. This phenomenom, which appears to be dependent on the period of the year of eye harvesting, can be induced by the generation of activated oxygen species, and may result from intramolecular modifications of the protein.

## MATERIALS AND METHODS

Materials. NADPH was obtained from Boehringer. Ammonium sulphate and all inorganic chemicals were of reagent grade from BDH. Xanthine oxidase (EC 1.2.3.2) (1.0 U/mg) and all other biochemicals were purchased from Sigma Chemical Co.. Calf eyes were obtained from freshly slaughtered animals at the local slaughterhouse and the lenses were removed and kept frozen until needed.

Preparation of lens crude extract. Frozen lenses were suspended (1 g/10 ml) in 50 mM sodium phosphate buffer, pH 6.8, supplemented with 5 mM 2-mercaptoethanol and homogenized at 4°C in a Potter-Elvehjem homogenizer. The suspension was centrifuged at 4°C for 30 min at 40,000 x g. The supernatant was subjected to ultracentrifugation at 100,000 x g at 4°C for 40 min. The supernatant which contains 10.0 $\pm$ 2.8 mU/ml (25 subjects), expressed as a mean $\pm$ SD and an average of 16 mg/ml protein, is referred to as "crude extract".

Enzyme activity measurement. Assay of enzyme activity was performed at 37°C in a reaction mixture (0.5 ml final volume) containing 90 mM potassium phosphate buffer, pH 6.8, 0.38 M ammonium sulphate, 4.7 mM DL-glyceraldehyde and 0.11 mM NADPH. The reaction was initiated by addition of the substrate and activity was measured in a DU-6 Beckman spectrophotometer by following the decrease in absorbance at 340 nm which parallels the coenzyme oxidation. The rate of decrease in absorbance in a parallel assay in which the substrate was omitted was subtracted as a blank to the enzyme activity determinations. Differences were not observed either on blank measurements or on the final enzyme activity when the reaction was initiated by addition of the crude extract, instead of the substrate.
One unit of enzyme activity is the amount of enzyme that catalyzes the oxidation of 1 $\mu$mol of NADPH/min.

Other methods. Protein was determined according to Bradford (12), by using bovine serum albumin as standard.

## RESULTS AND DISCUSSION

An increase of aldose reductase activity up to 3 times the initial value can be observed when crude bovine lens extracts are in-

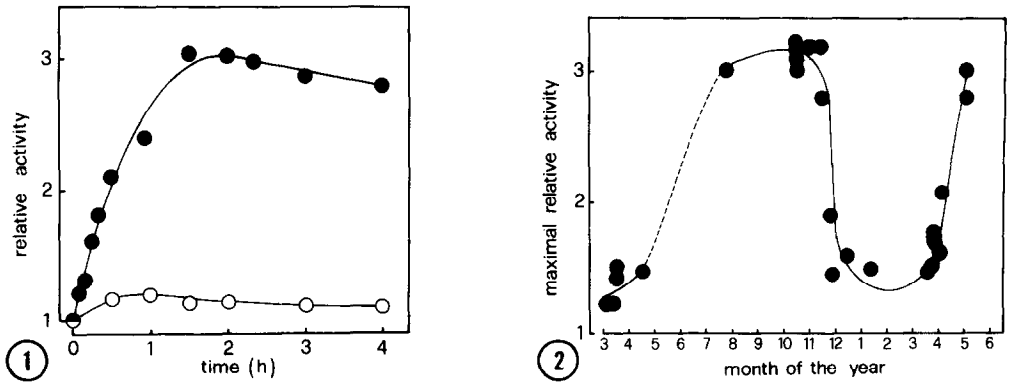


FIGURE 1. Time curve of activation of aldose reductase in lens crude extracts of calves sacrified in December (○) and in August (●). The incubation mixture contained in a final volume of 1.5 ml, 1.0 ml of crude extract, 85 mM sodium phosphate, pH 6.8, and 0.42 M ammonium sulphate (standard incubation mixture). The mixture was incubated at 37°C and at the indicated times, 75 μl were withdrawn and aldose reductase was measured as described in "Materials and Methods". The relative activity is the ratio of the activity measured at the indicated times of incubation to that determined at the initial time. The initial activity of the two extracts was 11 mU/ml and 9.2 mU/ml for winter and summer lenses, respectively.

FIGURE 2. Seasonal dependence of activation of bovine lens aldose reductase. The different susceptibility to activation of aldose reductase of lenses harvested at the indicated months of the year, is expressed as the ratio of the maximal activity measured after incubation of the extract in the standard mixture (see Fig. 1) compared to the activity determined at the initial time.

cubated at 37°C at relatively high ionic strength. The ability of the enzyme to be activated is dependent on the period of the year in which eyes are harvested. This is shown in Fig. 1 in which the increase in activity of aldose reductase of an extract of a lens collected in August, contrasts with the lack of activation of the enzyme of a lens collected in December. The seasonal occurrence of the activation process, evaluated as maximal relative activity, is reported in Fig. 2. A relative high ionic strength in the incubation medium appears to be necessary in order to activate the enzyme. Ammonium sulphate at concentrations ranging between 0.1 and 0.4 M allows a full activation of the enzyme. Ammonium sulfate can be replaced by different salts, such as magnesium sulfate and potassium chloride, present at a comparable ionic strength. Addition of several compounds: 1 mM phenylmethylsulphonylfluoride,

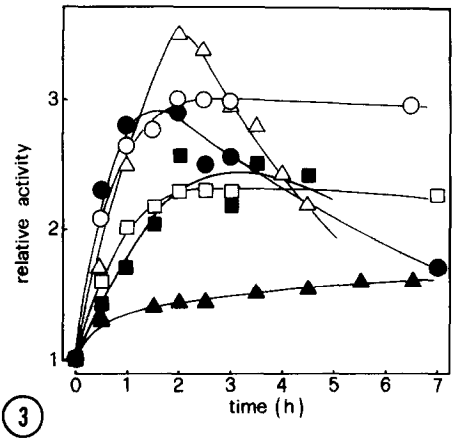


FIGURE 3. Activation of aldose reductase from winter lenses by systems known to generate oxygen activated species. The incubation mixture was as indicated in Fig. 1. The following effectors were added: (▲) none; (●) 0.1 mM $FeSO_4$ + 0.3 mM EDTA; (○) 0.1 mM $FeSO_4$ + 0.3 mM EDTA + 10 mM mannitol; (□) 0.1 mM $FeSO_4$ + 0.3 mM EDTA + 1 mM sodium formate; (△) 0.1 mM $FeSO_4$ + 0.3 mM EDTA + 1 mM Hyp + 0.02 U/ml xanthine oxidase; (■) 1 mM Hyp + 0.02 U/ml xanthine oxidase.

FIGURE 4. Time curve of activation of aldose reductase in the presence of different concentrations of reduced glutathione. An extract of a lens harvested in August was incubated in the standard mixture (see Fig. 1) containing 0.1 mM $FeSO_4$, 0.3 mM EDTA and 0 (○), 1 (●), 2 (▲), 5 (■) and 10 (□) mM reduced glutathione. The activation of the enzyme in the absence of the $FeSO_4$/EDTA system is also reported (△).

0.55 mg/ml trypsin inhibitor, 1 mM EDTA, 1 mM ATP, 60 $\mu$M histamine, 1.5 mM putrescine, 1 mM calcium chloride, did not affect the activation process.

Oxygen activated species already shown to cause oxidative modifications of lens crystallins (13,14), appear to be involved in the modulation of aldose reductase activity. This is shown in Fig. 3, where systems known to generate oxygen radicals such as $Fe^{+2}$/EDTA or Hyp/xanthine oxidase, induce the enhancement of the enzyme activity in an extract in which high ionic strength alone fails to activate the enzyme (winter lenses, Fig.2).

Following the activation process, a rather slow decline of aldose reductase activity is evidenced upon prolonged incubation (Fig. 3). This decline, which is more pronounced when the above activating systems are both present, appears to be prevented by

addition of radical scavengers to the incubation mixture. Mannitol, which seems not to affect the activation process of aldose reductase, is very effective in preserving the activated state of the enzyme. Also formate can prevent the activity decline; however, it appears to affect the amplitude and the rate of activation.

Reduced glutathione added to the incubation mixture strongly impairs aldose reductase activation, with a $I_{50}$ (concentration of glutathione which determines a decrease of 50% in maximal activation) of 2.5 mM (Fig.4). Dithiothreitol, at a final concentration of 10 mM, completely inhibits the activation process. Owing to the presence of 2-mercaptoethanol in the buffer used for the preparation of the lens crude extract, this thiol compound is always present in the incubation mixture at a final concentration of 3 mM. Supplementary 2 mM 2-mercaptoethanol, added to the incubation mixture does not affect the capability of the enzyme to be activated, whereas a decrease in the rate of activation is observed when the thiol compound is present at a final concentration of 15 mM.

A protein-protein dissociation reaction, induced by high ionic strength, similar to that reported for human placenta, liver and kidney aldehyde reductase (15-17), cannot be excluded as a possible step in the activation phenomenon. In the case of human tissues, the $\alpha$-subunit generated by dissociation of $\alpha,\beta$-dimeric aldehyde reductase at high ionic strength is an aldose reductase. However, the seasonal dependence of activation of bovine lens aldose reductase and the marked influence of oxygen radicals and thiol compounds is indicative that a more complex molecular process is involved in the activation of the enzyme.

Even though radicals can interact with proteins in different ways, the prevention of aldose reductase activation exerted by glutathione and other thiol compounds suggests that the reduced/

oxidized state of specific SH-groups of the protein might be involved in defining different enzyme forms.

Erythrose, as well as glyceraldehyde, can be used as a substrate to follow the enhancement of aldose reductase activity, while the use of glucose, xylose and glucuronate, does not reveal any activation of the enzyme. The autoxidation of glyceraldehyde and other monosaccharides has been invoked to explain the non-linear kinetics of aldose reductase (9). In our experimental conditions, changes in the composition of low molecular weight species in the medium during incubation might enhance the autoxidation of the substrate as well, with subsequent apparent enhancement of the enzyme activity. However, this possibility can be ruled out for the following reasons: i) both activated and non-activated enzyme preparations retain their activities after dialysis, ii) no changes in aldose reductase activity was observed when dialyzed activated and non-activated enzyme preparations were cross-combined with the respective ultrafiltrates (Amicon YM30 membranes), iii) an almost complete recovery of aldose reductase activity after gel filtration chromatography is obtained either from activated or non-activated extracts. These results suggest that the activation phenomenon must be related to modifications of aldose reductase and/or of undialyzable species present in the extract, that elute with the enzyme on molecular sieving chromatography.

Even though our evidence of aldose reductase activation does not permit us to define a general model of the enzyme activity modulation, it could be a signal of unsuspected regulatory properties of this protein. Moreover, the seasonal occurrence of the activation phenomenon (Figs 1 and 2) is indicative of modifications in the lens which could modulate <u>in vivo</u> the activity of aldose reductase.

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