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AMIDINATION OF AMINO GROUPS OF ALDEHYDE REDUCTASE FROM HUMAN LIVER

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Summary

Amidination of human liver aldehyde reductase (alcohol:$NADP^+$ oxidoreductase, EC 1.1.1.2) with monofunctional *n*-alkane methylimidates increased the enzymic activity by 10—30%, whereas analogous bifunctional imidoesters caused a loss of activity of about 80%. Both effects were prevented in the presence of the coenzyme NADPH or $NADP^+$, but not of the substrate 4-nitrobenzaldehyde. Amidination increased the apparent Michaelis constant of both the coenzyme (up to 20-fold) and the substrate (about 5-fold). Bifunctional imidoesters with at least 4 carbon atoms between the functional groups (approx. 0.7 nm) crosslinked the enzyme intramolecularly. This reaction was retarded in the presence of the coenzyme, whereas 4-nitrobenzaldehyde had no effect. The results suggest the presence of reactive amino groups at the coenzyme binding site of aldehyde reductase.

Introduction

Aldehyde reductase (alcohol: $NADP^+$ oxidoreductase, EC 1.1.1.2) is a monomeric pyridine nucleotide-dependent oxidoreductase catalyzing the reduction of a variety of aromatic, medium chain length aliphatic and uncyclized sugar aldehydes. Little is known about the structure and mechanism of this enzyme. We have recently purified the enzyme from human liver and shown that modification of cysteine residues abolished the enzymatic activity [1]. Modification

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of other amino acid residues of aldehyde reductase has not been reported. On the other hand there are reports implicating a lysine residue in the coenzyme binding domain of oligomeric aldehyde reducing enzymes [2—5]. In order to examine structural similarities between monomeric and oligomeric enzymes we investigated the effects of the amidination of amino groups [6] on the catalytic and structural properties of aldehyde reductase. Imidoesters do not interfere with cysteine residues and, since the resulting amidines are stronger bases than the parent amines, they do not change the net positive charge of the modified amino groups.

In this paper, we show that reactive amino groups are present in the coenzyme binding domain of human liver aldehyde reductase. A preliminary account of this study has been presented [7].

Materials and Methods

Enzyme

Aldehyde reductase was prepared from human liver as described previously [1]. The protein concentration was determined on the basis of the absorption coefficient: $\epsilon_{280} = 54\ 000\ M^{-1} \cdot cm^{-1}$ [1].

Imidoesters

Acetic methylimidate was purchased from Pierce, Rockford, U.S.A. The other monofunctional imidoesters were prepared from the corresponding nitriles according to Plapp and coworkers [2]. Valeronitrile, capprononitrile and caprylonitrile were purchased from Fluka, Buchs, Switzerland. The diimidoester hydrochlorides were synthesized from the corresponding dinitriles according to McElvain and Schroeder [8]. The quality of the synthesized reagents was tested with rabbit muscle aldolase [9]. All diimidoesters gave the expected electrophoretic patterns. Malononitrile was obtained from Fluka, Buchs, Switzerland, glutaronitrile, adiponitrile and suberonitrile from Merck-Schuchardt, Darmstadt, F.R.G. All imidoester hydrochlorides were stored in the cold over P_2O_5 and NaOH and used within two months after synthesis.

Amidination

Reactions with imidoesters were routinely carried out at room temperature (20°C) in 0.2 M triethanolamine-Cl (pH 8.5). Aldehyde reductase was used at 0.4—0.5 mg/ml (11—14 μM). At intervals, aliquots were withdrawn from the reaction mixtures. In samples used for electrophoresis, the reaction was stopped by the addition of 1/10 vol. 1 M hydroxylamine. Aliquots used for activity measurements were directly added to the assay mixture. No influence of unreacted imidoesters or any other component of the reaction mixture on the assay for enzymic activity was observed under these conditions.

Enzyme assay

Aldehyde reductase activity was assayed by recording the decrease of NADPH absorbance as a function of time at 340 nm. The standard reaction mixture contained in a total volume of 1 ml: 0.08 M sodium phosphate buffer (pH 7.0), 0.08 mM NADPH and 0.5 mM 4-nitrobenzaldehyde. The reaction was

started by the addition of enzyme. One enzyme unit is defined as the oxidation of 1 μmol NADPH per min.

Polyacrylamide gel electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis and staining with Coomassie brilliant blue R-250 were performed according to Fairbanks et al. [10]. Aliquots containing 2—4 μg protein were applied to the gels. Densitometric evaluation of gels was performed on a Gelman ACD-15 densitometer. As assessed by calibration, the plot of density versus amount of protein was linear up to 4 μg protein per band.

Determination of free amino groups

Aldehyde reductase was amidinated as described above. Excess reagent and triethanolamine were removed by extensive dialysis against 2.5 mM sodium tetraborate and determination of free amino groups was carried out by trinitrophenylation in the presence of 6 M guanidinium chloride as described by Fields [11]. The percentage of free amino groups was calculated from the difference of ΔA_{420}/mg protein between the native (100%) and the amidinated protein.

Results

Modifications with monofunctional imidoesters

Aldehyde reductase was treated with monofunctional aliphatic imidoesters of different chain lengths. As shown in Figs. 1 and 2 they changed the enzymatic activity by a process dependent on the reagent concentration and on the time of incubation. Limited modification increased the activity by 10 to 30% but further amidination reversed this activation or even partially inactivated the enzyme. At low concentrations of the modifying agent more activa-

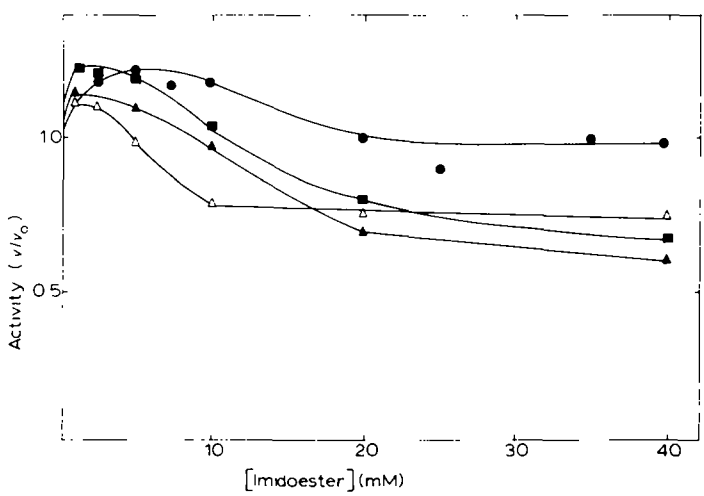


Fig. 1. Effect of monofunctional imidoesters on the activity of human liver aldehyde reductase. Enzyme was treated with incrasing concentrations of (△) acetic (▲) valeric (■) capronic and (●) caprylic methylimidate. After 60 min, aliquots were withdrawn from the incubation mixture and assayed for activity. v_0 represents initial velocities in the absence and v in the presence of imidoester.

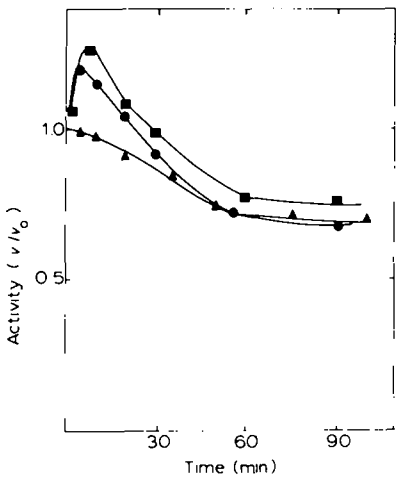


Fig. 2. Time dependence of amidination of aldehyde reductase by monofunctional imidoesters. Enzyme was incubated with 20 mM capronic methylimidate and aliquots were withdrawn from the reaction mixture after the times indicated in the graph. (●) Control, (▲) in the presence of 0.5 mM NADPH, (■) in the presence of 0.5 mM 4-nitrobenzaldehyde.

tion was observed with the more hydrophobic imidoesters capronic and caprylic methylimidate. In order to assess the effect of the coenzyme (NADP(H)) and substrate (4-nitrobenzaldehyde) on the modifications, amidinations were carried out in the presence of these ligands. The reduced as well as the oxidized coenzyme, buth not the substrate, prevented the activation, whereas little influence was observed on the partial inactivation by high imidoester concentrations (Fig. 2).

Prolonged modification over 4 h with a low (2 mM) and high (10 mM) concentration of capronic methylimidate led to an activation and inactivation of the enzyme, respectively. The degree of the alteration of activity was dependent on the concentrations of coenzyme and substrate in the assay. Under standard assay conditions (80 μM NADPH, 0.5 mM 4-nitrobenzaldehyde), treatment with 2 mM capronic methylimidate increased the activity by 13%, whereas incubation with 10 mM imidate decreased it to 65% of the control. If the assays were carried out at a 4-fold and 2-fold concentration of coenzyme and substrate, respectively, the activation of the enzyme by 2 mM capronic methylimidate was 18% and the enzyme modified with 10 mM imidoester showed the same activity as the control. These findings suggested that under standard assay conditions the enzyme was saturated to a lower degree than the native enzyme. The results in Table I show that modification with increasing concentrations of capronic methylimidate indeed increased the Michaelis constants for NADPH (approx. 20-fold) and for 4-nitrobenzaldehyde (approx. 5-fold). However, as indicated by the maximal velocity, the activity of the enzyme modified with 40 mM capronic methylimidate remained decreased in comparison to the native enzyme even at saturating coenzyme and substrate concentrations. An estimate of the degree of amidination was obtained by titration of unreacted amino groups with trinitrobenzene sulfonate. Treatment with 2 mM and 20 mM capronic methylimidate modified 20 S.D. ± 5.8% (n = 4)

TABLE I

INFLUENCE OF AMIDINATION ON THE KINETIC CONSTANTS OF ALDEHYDE REDUCTASE

Aldehyde reductase was incubated for 4 h with capronic methylimidate at the concentrations indicated. The values for the Michaelis constants for the coenzyme ($K_{m,NADPH}$) and the substrate, 4-nitrobenzaldehyde ($K_{m,pNB}$), and the maximal velocity (V) were obtained from replots of the intercepts and slopes of double reciprocal plots versus the reciprocal concentrations of the fixed substrate. The concentrations of NADPH and pNB were varied between 10 and 200 μM and 80 and 500 μM, respectively.

Concn. of capronic methylimidate (mM)	$K_{m,NADPH}$ (μM)	$K_{m,pNB}$ (μM)	V (U/mg)
0	2.5	114	14.3
2	14	222	18.3
10	37	268	14.6
40	55	570	11.4

and 35 S.D. ± 13% (n = 3) of the amino groups, respectively. Since aldehyde reductase contains 20 lysine residues [1] these percentages correspond to about 4 and 7 modified amino groups, respectively.

Modifications with bifunctional imidoesters

Effects on the activity. Fig. 3 shows the effect of bifunctional imidoesters on the activity of aldehyde reductase. Contrary to the activation observed with monofunctional imidoesters, bifunctional reagents decreased the activity even at low concentrations. An exception was observed with malonic dimethylimidate which did not markedly affect aldehyde reductase activity at low concentrations. Progress curves of inactivation (Fig. 4) show that NADPH prevented inactivation during the first 10 to 20 min and retarded it in the further course of the reation. No protection was observed with 4-nitrobenzaldehyde. Treatment of aldehyde reductase with 10 mM suberic dimethylimidate increased the apparent K_m values for NADPH and 4-nitrobenzaldehyde to 21 and 450 μM, respectively.

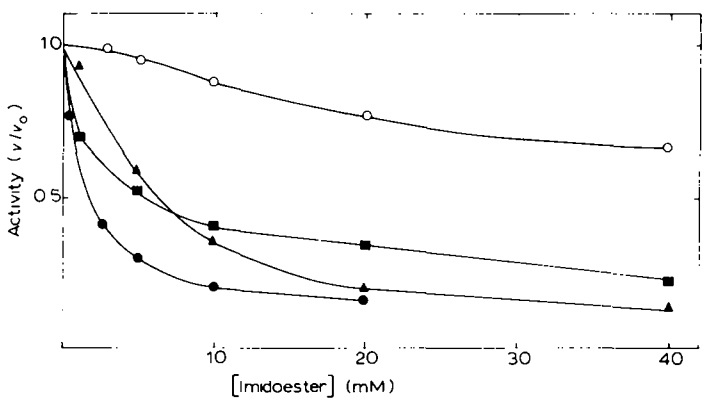
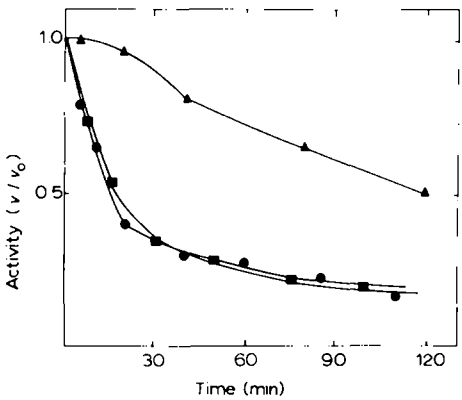


Fig. 3. Inactivation of human liver aldehyde reductase by bifunctional imidoesters. Enzyme was treated with increasing concentrations of (○) malonic (▲) glutaric, (■) adipic and (●) suberic dimethylimidate. After 90 minutes aliquots were withdrawn from the reaction mixture and assayed for activity.



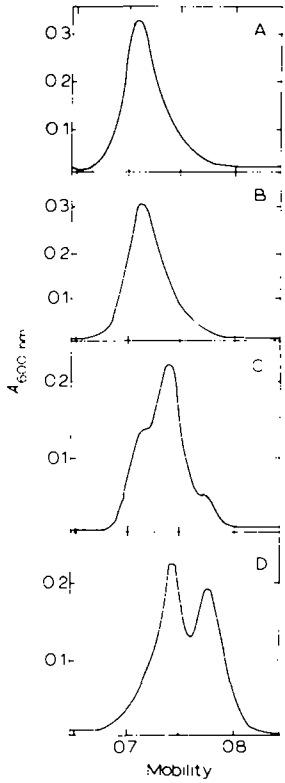


Fig. 4. Time dependence of inactivation of aldehyde reductase by bifunctional imidoesters. Enzyme was incubated with 10 mM suberic dimethylimidate and aliquots were assayed for activity after the times indicated in the figure. (●) Control, (▲) in the presence of 0.5 mM NADPH, (■) in the presence of 0.5 mM 4-nitrobenzaldehyde.

Fig. 5. Crosslinking of aldehyde reductase by bifunctional imidoesters. Enzyme was incubated with 10 mM (B) glutaric, (C) adipic and (D) suberic dimethylimidate. (A) represents a control in the absence of imidoester.

Effects on the structure

Bifunctional imidoesters are able to crosslink proteins either inter- or intramolecularly provided that two amino groups are present within the distance of the reaction groups. Aldehyde reductase was treated with diimidoesters of different crosslinking distances (d) and subjected to sodium dodecylsulfate polyacrylamide gel electrophoresis.

Fig. 5 shows the densitometric scans of the corresponding disc electrophoretic patterns. One protein band was observed when the enzyme was incubated with reagents of 3 or less carbon atoms between the imidate functions ($d \leqslant 0.61$ nm). On the other hand, 3 bands were obtained with diimidoesters having 4 or more carbon atoms between the imidoester groups ($d \geqslant 0.73$ nm). By comparison of relative mobilities the slowest migrating band could be assigned to the non-crosslinked enzyme. The two other bands most likely correspond to intramolecularly crosslinked enzyme forms which are retained in a partially folded structure by the new bonds in the presence of sodium dodecylsulfate and thus migrate according to a lower apparent molecular

TABLE II

EFFECT OF NADPH AND TIME OF INCUBATION ON THE CROSSLINKING OF ALDEHYDE REDUCTASE

Aldehyde reductase was treated with 10 mM suberic dimethylimidate in the presence and absence of 0.5 mM NADPH. Aliquots were withdrawn from the reaction mixture after 10 and 60 min and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis. The fraction of each enzyme form was quantitated by densitometry of the gels.

Enzyme form	Percentage of total protein			
	— NADPH		+ NADPH	
	10 min (%)	60 min (%)	10 min (%)	60 min (%)
Non-crosslinked enzyme	26	15	57	23
Crosslinked form 1 *	55	41	30	39
Crosslinked form 2 *	19	44	13	38

* Form 1 corresponds to the slower moving, form 2 to the faster moving crosslinked enzyme (see Fig. 5).

weight than the completely unfolded native enzyme. The formation of crosslinks was dependent on the time of incubation and was retarded by NADPH. This is illustrated by the results in Table II.

Protein bands corresponding to intermolecularly crosslinked enzymes could not be detected.

Discussion

The results of this study indicate that human liver aldehyde reductase contains two classes of amino groups which after modification change the catalytic activity of the enzyme. The existence of at least two reactive amino groups, about 0.7 nm apart, at the coenzyme binding domain of the enzyme is suggested by the activation through monofunctional and inactivation through bifunctional imidoesters and the protection against these effects furnished by the coenzyme, as well as by the results from the crosslinking experiments. Their role could be to act as binding sites for the negatively charged pyrophosphate and 2'-phosphate moiety of the coenzyme, a possibility which is supported by the finding that succinylation, which introduces a negative charge, completely inactivated the enzyme (unpublished data).

The second class of amino groups is modified by higher imidoester concentrations and the reaction is not prevented or retarded by the coenzyme. The increase of the Michaelis constants produced by modification of these amino groups may rather be due to a general perturbation of the tertiary structure than to the modification of a specific amino acid residue in the active site of the enzyme.

The diverging effects of monofunctional and bifunctional imidoesters on the activity of aldehyde reductase are best explained by the assumption that the hydrophobic tails of the monofunctional reagents occupy different positions in the coenzyme binding site than the more hydrophilic residues of the bifunctional imidoesters. The results obtained with glutaric dimethyl imidate show

that the inactivation by bifunctional imidoesters appears not to be caused by the crosslinking reaction proper. It is rather the result of any (hydrophilic) interaction of the second imidate function or its hydrolysis products with the enzyme, forcing the bridging hydrophobic methylene groups into a catalytically unfavorable position. Comparisons of our results with those obtained with other aldehyde reducing enzymes show similarities of aldehyde reductase and alcohol dehydrogenase from human and horse liver [2,3]. These enzymes are activated up to 10-fold by monofunctional imidoesters. It must be noted, however, that such rates of activation were obtained at very high substrate concentrations and that under conditions similar to ours the activation was only 1.2-fold. Kinetic studies have shown that the increased activity of the modified alcohol dehydrogenase is due to an enhanced rate of dissociation of NADH, the rate limiting step of alcohol oxidation.

NADP-dependent retinol dehydrogenase from bovine rod outer segments is inactivated by acetic methylimidate but activated by picolinic methylimidate [12]. The results indicate, however, that the active amino group may be part of the substrate rather than the coenzyme binding site.

The effects of amino group modification of yeast alcohol dehydrogenase on the enzymic activity are little investigated. It appears, however, that acetimidylation completely inactivates the enzyme [4].

Glyceraldehyde-3-phosphate dehydrogenase from rabbit muscle is inhibited by several amino group modifying agents [5]. Structural analysis has shown that at least 3 lysine residues are specifically modified and that two of them are protected against modification by the coenzyme.

These comparisons show that human liver aldehyde reductase resembles other aldehyde reducing enzymes with respect to the presence of amino groups in the active site, It remains, however, to be clarified in how far these amino groups are related to a structural homology in these enzymes.

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