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THE REACTION MECHANISM OF ALDOSE REDUCTASE FROM RHODOTORULA

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SUMMARY

Aldose reductase (alditol:NADP$^+$ oxidoreductase, EC 1.1.1.21) from Rhodotorula catalyzes an essentially irreversible oxidation of NADPH by glyceraldehyde at pH 7.5. At pH 9.0 the reverse reaction could be demonstrated. Initial velocity and product inhibition data indicate that the reaction mechanism at pH 7.5 is Ordered Bi Bi while at pH 9.0 it is Random Bi Bi. Activation of the reverse reaction by high glycerol concentrations was observed. Substrate activation of the forward reaction could not be demonstrated. Multivalent anions were observed to competitively inhibit the forward and activate the reverse reactions. A model is suggested which offers a unified explanation for changes in reversibility, reaction mechanism and effects of multivalent anions as well as substrate activation.

INTRODUCTION

Aldose reductase (alditol:NADP$^+$ oxidoreductase, EC 1.1.1.21), from a number of mammalian tissues as well as some microorganisms has been described[1-12]. The reaction catalyzed is the following:

$$\text{Aldose} + \text{NADPH} + \text{H}^+ \rightleftharpoons \text{polyol} + \text{NADP}^+$$

A variety of aldoses may act as substrate; however, the cofactor requirement is specifically for NADP.

Sulfate has been shown to be an activator of some aldose reductases and an inhibitor of others. HASTEIN AND VELLE[2] observed that sulfate activated the forward reaction (NADPH oxidation) and inhibited the reverse reaction catalyzed by the enzyme from placenta and seminal vesicles. They also reported substrate (glyceraldehyde) activation at high concentrations, as did THRASH[13] for the lens enzyme. The reaction mechanism for aldose reductase partially purified from rat skeletal

Abbreviation: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

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muscle has been reported by TOWES[14]. Reaction mechanism studies of a highly purified aldose reductase from any source have not been previously reported. Therefore, the observations concerning the sulfate effect have gone without interpretation while substrate activation was assumed to occur in the same manner as was reported for the liver alcohol dehydrogenase system at high alcohol concentrations[15].

The present study describes the reaction mechanisms of aldose reductase from Rhodotorula at both pH 7.5 and 9.0. The mechanisms are used as a model to explain both activation and inhibition by multivalent anions as well as substrate activation described in this report for the Rhodotorula system. The model also offers a unified explanation for some of the divergent properties reported previously by others for aldose reductase from various tissues.

EXPERIMENTAL PROCEDURE

Materials. DL-Glyceraldehyde, NADPH and $NADP^+$ were purchased from Sigma. Glycerol, Na_2SO_4 and $Na_4P_2O_7$ were from Fisher. $(NH_4)_2SO_4$ was obtained from Mann.

Enzymatic assay. The standard assay used for aldose reductase was described in a previous report[12]. Substrates and modifiers were made fresh at the beginning of each experiment in *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)–NaCl buffer (0.05 M HEPES and 0.10 M NaCl, pH 7.5) or in Tris–HCl buffer (0.10 M Tris, pH 9.0) and kept at 0°. These reagents were added to the cuvette first and equilibrated at 37° before the buffer (kept at 37°) was added to a final volume of 0.975 ml. All reactions were initiated with 25 μl of the highly purified enzyme preparation[12].

RESULTS

The pH optimum for NADPH oxidation by glyceraldehyde (the forward reaction) was 7.5. When $NADP^+$ and glycerol were incubated with the enzyme at pH 7.5 no reduction of $NADP^+$ (the reverse reaction) could be detected. Initial velocities of the forward reaction were studied at pH 7.5 by varying the concentra-

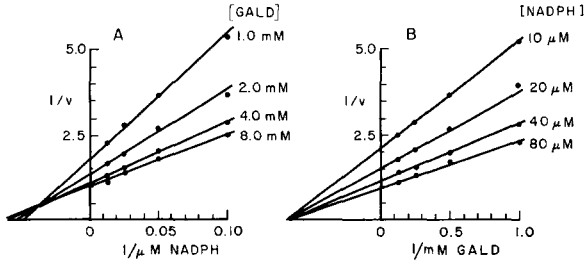

Fig. 1. Initial velocity patterns at pH 7.5. Double reciprocal plots of: (A) Varying NADPH concentration with DL-glyceraldehyde (GALD) at 1.0 mM, 2.0 mM, 4.0 mM and 8.0 mM. The point of intersection to the left of the ordinate represents the negative reciprocal of the dissociation constant of NADPH from the *E*–NADPH binary complex[24]. The value of this dissociation constant is 23 μM. (B) Varying DL-glyceraldehyde (GALD) concentrations with NADPH at 10 μM, 20 μM, 40 μM and 80 μM.

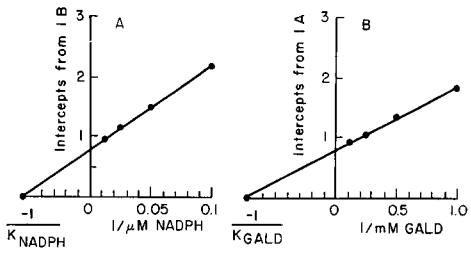

Fig. 2. Secondary plots from Fig. 1. (A) Intercepts from Fig. 1B *versus* the reciprocal of the NADPH concentration. (B) Intercepts from Fig. 1A *versus* the reciprocal of the glyceraldehyde (GALD) concentrations. The limiting values of K_{NADPH} and K_{GALD} at pH 7.5 determined by this method were $1.8 \cdot 10^{-5}$ M and $1.35 \cdot 10^{-3}$ M.

tion of one substrate while the other was kept constant with no reaction products added. The results are plotted in Fig. 1. In both sets of experiments the lines converge upon a point to the left of the ordinate. These patterns indicate that no irreversible step (such as release of a product not present initially) occurred between attachment of the first and second substrate to the enzyme and are characteristic of a sequential mechanism, *i.e.* both substrates add to the enzymes before any products are released[16]. Under the standard assay conditions the limiting Michaelis constant for NADPH was $1.8 \cdot 10^{-5}$ M (Fig. 2A) and that for DL-glyceraldehyde was $1.35 \cdot 10^{-3}$ M (Fig. 2B), with both D- and L-glyceraldehyde able to serve as substrates.

The product cofactor, $NADP^+$, was found to be an inhibitor of the forward reaction[12]. The product inhibition pattern of $NADP^+$ is of the following type: competitive when NADPH is varied (at all concentrations of glyceraldehyde), non-

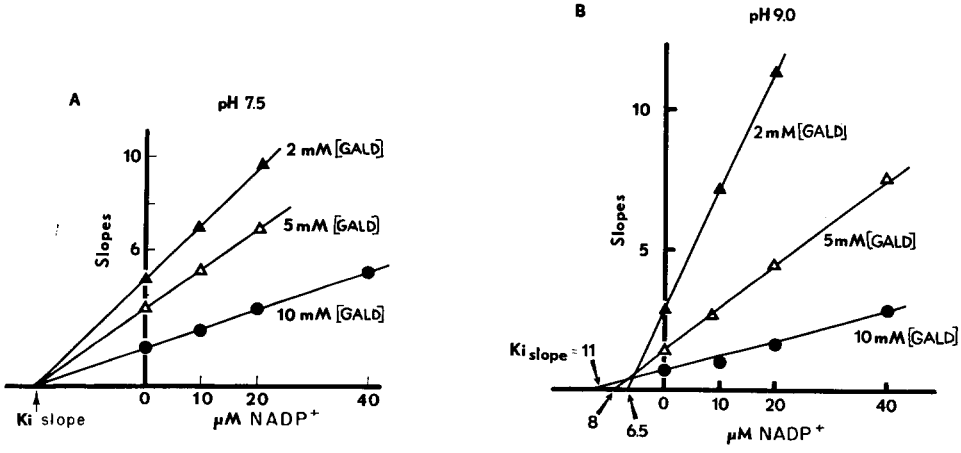

Fig. 3. Determination of K_i slope for $NADP^+$ at (A) pH 7.5 and (B) pH 9.0. Plots of $1/v$ *vs.* $1/\mu M$ NADPH were made at three levels of DL-glyceraldehyde (GALD) with $NADP^+$ concentrations ranging from 0 to 40 μM. The slopes of the resulting lines are plotted above *vs.* μM $NADP^+$, and from the best statistical curve the value of K_i slope for each concentration of DL-glyceraldehyde was determined. (A) At pH 7.5, K_i slope was constant at 20 μM for all three glyceraldehyde concentrations tested. (B) At pH 9.0, K_i slope varied from 11 μM (at 10 mM DL-glyceraldehyde) to 6.5 μM (at 2 mM DL-glyceraldehyde).

competitive when glyceraldehyde is varied (at non-saturating concentrations of NADPH) and no inhibition when NADPH is saturating and glyceraldehyde is varied. Such inhibition indicates, but does not distinguish between, an ordered or rapid equilibrium random mechanism[17]. Mathematical considerations of Bi Bi mechanisms would allow a distinction to be made between these two types on the basis of the inhibition pattern of the other product, glycerol[17]. However, in this system at pH 7.5 glycerol exhibited no inhibitory properties of any type. According to CLELAND, these two mechanisms can be distinguished by determining K_i slope (for $NADP^+$) at different concentrations of glyceraldehyde: a constant K_i slope indicating an ordered mechanism, and a changing K_i slope indicating a random mechanism[18]. Initial velocities were studied at 2.0 mM, 5.0 mM and 10 mM glyceraldehyde at various $NADP^+$ levels with NADPH as the variable substrate. Double reciprocal plots were made and the slopes plotted *versus* $NADP^+$ concentration for each level of glyceraldehyde. The results (Fig. 3A) show the K_i slope to be constant under the conditions investigated and together with the $NADP^+$ inhibition data support an ordered mechanism in which NADPH added first to the free enzyme. A ternary complex was then formed by the attachment of glyceraldehyde, and the conversion to products took place. The first product, glycerol, was released; $NADP^+$ then dissociated from the enzyme–$NADP^+$ complex, regenerating the free enzyme.

It was found that this enzyme could catalyze $NADP^+$ reduction by glycerol at basic pH values. This together with the observation that glycerol inhibited the forward reaction only at pH 9.0 indicated that the reaction mechanism at the higher pH may differ from that at pH 7.5. Initial velocity patterns for the forward reaction at pH 9.0 indicated that the mechanism was sequential. A determination of K_i slope for $NADP^+$ at the three levels of glyceraldehyde used previously at pH 7.5 (2.0 mM, 5.0 mM and 10 mM) showed that K_i slope now increased 70% with increasing glyceraldehyde concentrations (Fig. 3B). At pH 9.0 glycerol inhibited the forward reaction in a manner which, according to CLELAND[17], was indicative of a Random Bi Bi reaction mechanism; namely, glycerol was a competitive inhibitor of both NADPH and glyceraldehyde (when the substrate held constant was non-saturating). These results support a random mechanism in which either substrate may add first to the enzyme and either product may be released first.

Kinetics of the reverse reaction at pH 9.0

Double reciprocal plots of initial velocities at $NADP^+$ concentrations ranging from 1 μM to 240 μM, with 200 mM glycerol present in all reactions were linear with an apparent K_m of 10 μM $NADP^+$. A similar plot, at 125 μM $NADP^+$, with glycerol concentrations from 2 mM to 200 mM showed a marked downward curvature at the higher substrate concentrations (Fig. 4, Control Curve). Two apparent K_m values for glycerol were extrapolated from the linear portions of the curve[19]: K_m = 4.5 mM, at low glycerol concentrations; and K_m = 40 mM at higher glycerol concentrations. In the forward reaction with glyceraldehyde as the variable substrate at both pH 7.5 and 9.0, no substrate activation was observed over the range of glcyeraldehyde concentrations examined (0.1 mM to 80 mM).

The effect of multivalent anions

Initial velocities of the forward reaction were studied at pH 7.5 in the

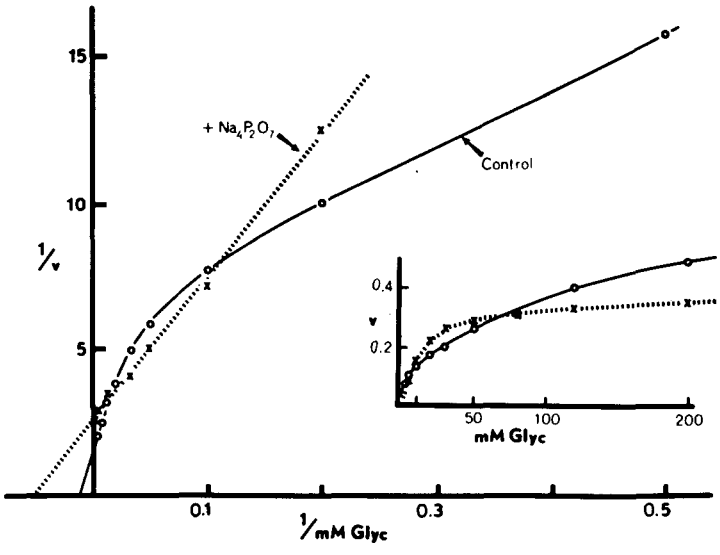

Fig. 4. Double reciprocal plots of initial velocities *vs.* glycerol (Glyc) concentration, at pH 9.0, in the presence and absence of $Na_4P_2O_7$. The standard assay was used with $NADP^+$ at 125 μM in all reactions. Glycerol concentrations ranged from 2 mM to 200 mM, and $Na_4P_2O_7$ was at 50 mM (dotted lines). No pyrophosphate was added to control reactions (solid lines). Two values for K_m were extrapolated from the linear portions of the control curve: at low glycerol concentrations, K_m = 4.5 mM; at high glycerol concentrations, K_m = 40 mM. The insert shows the shape of the initial velocity *vs.* mM glycerol curves.

absence and presence of two anions. Purely competitive inhibition was exhibited by 100 mM $(NH_4)_2SO_4$ (K_i = 44 mM) and 20 mM $Na_4P_2O_7$ (K_i = 21 mM) when NADPH was the varied substrate. Both sodium and ammonium sulfate gave the same K_i.

Table I shows the results of initial velocities of the reverse reaction at pH 9.0 with 20 mM glycerol present in all reactions. In the absence of added multivalent anions, the apparent K_m for $NADP^+$ was 8.8 μM. The K_m increased to 31 μM as the concentration of sulfate was increased to 40 mM, while the maximum

TABLE I

ACTIVATION OF THE REVERSE REACTION BY MULTIVALENT ANIONS AT pH 9.0

The standard assay procedure was used with 20 mM glycerol in all reactions and $(NH_4)_2SO_4$ or $Na_4P_2O_7$ as indicated. $NADP^+$ concentrations were varied from 10 μM to 120 μM. The kinetic constants were determined from double reciprocal plots. Maximum velocities are expressed as μM $NADP^+$ reduced per min.

Effector	K_m *(app) for* $NADP^+$ *(μM)*	v_{max} *(μM/min)*	% v_{max} *inc.*	K_i *(mM)*
None	8.8	208	—	—
4 mM SO_4^{2-}	12	229	10	13
10 mM SO_4^{2-}	15	251	22	13
40 mM SO_4^{2-}	31	283	38	15
20 mM $P_2O_7^{4-}$	25	328	60	11

velocities increased 30% over the same range of sulfate concentrations. The competitive K_i for sulfate was about 14 mM. The effect of 20 mM $Na_4P_2O_7$ was similar: the apparent K_m for $NADP^+$ increased to 25 μM and the maximum velocity increased 60%. The competitive K_i for pyrophosphate was 11 mM. The presence of 50 mM $Na_4P_2O_7$ abolished substrate activation by glycerol (Fig. 4, dotted line).

DISCUSSION

The reaction at pH 7.5

The initial velocity and product inhibition data for the forward reaction at pH 7.5 indicated that the mechanism was Ordered Bi Bi as shown in Fig. 5, with the rate limiting step being the release of glycerol from the ternary complex. At pH 7.5 the equilibrium favors NADPH oxidation; however, at high concentrations of $NADP^+$ and glycerol the reduction of $NADP^+$ would occur to an extent which could easily be measured spectrophotometrically ($K_{eq} = 5.0 \cdot 10^{-3}$ at pH 7.5 (ref. 20)). In studying a similar reaction using aldose reductase from placental tissue, HASTEN AND VELLE[2] observed $NADP^+$ reduction at pH 7.0. When $NADP^+$ and glycerol were incubated with the enzyme, no reduction of $NADP^+$ was detected. Such irreversibility is

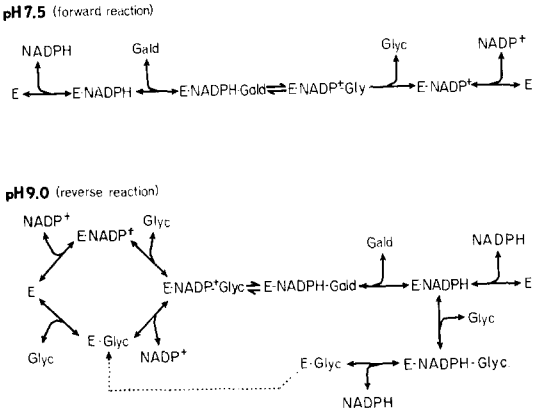

Fig. 5. The proposed reaction mechanisms at pH 7.5 and pH 9.0. The Ordered Bi Bi mechanism shown for the reaction at pH 7.5 is essentially irreversible at the point of release of glycerol from the ternary complex. At pH 9.0, the reaction is reversible and the mechanism is Random Bi Bi. The reverse reaction is shown here in order to demonstrate the proposed mode of substrate activation (which occurs only for the reverse reaction at high glycerol concentrations). Abbreviations: E, free enzyme; Gald, glyceraldehyde; Glyc, glycerol.

believed to be closely associated with the observation that glycerol failed to exhibit any effects upon the initial velocity of the forward reaction. The point of irreversibility is, therefore, believed to be the release of glycerol from the ternary complex as indicated in Fig. 5.

The reaction at pH 9.0

Studies of initial velocities and product inhibition for the forward reaction at pH 9.0 indicated that although the mechanism was still sequential, addition of substrates and release of products was random. The reaction was now reversible.

A very similar situation has been reported for the reaction mechanism of pig heart mitochondrial malate dehydrogenase. The mechanism was ordered at pH 8.0 (with the cofactor, NAD, being the first substrate to attach and last product to be released). At pH 9.0 the mechanism became partly random[21–23].

Substrate activation

Normal Michaelis–Menten kinetics have been observed for the reverse reaction at pH 9.0 when the concentration of $NADP^+$ was varied and glycerol held constant at 200 mM. However, when the glycerol concentration was varied and $NADP^+$ held constant (at 125 μM) a marked substrate activation occurred at higher levels of glycerol. This phenomenon has also been observed for the forward reaction with aldose reductase from placenta[2], seminal vesicle[2] and lens[13]. Similar results were also reported for liver alcohol dehydrogenase by DALZIEL AND DICKINSON[15]. These authors postulated that for alcohol dehydrogenase the rate limiting step was dissociation of the *E*–NADH binary complex. At high alcohol concentrations an abortive *E*–NADH–alcohol ternary complex could be formed from which NADH dissociated more rapidly. This reasoning is consistent with the random mechanism proposed for yeast aldose reductase at pH 9.0, and is presented in graphic form in Fig. 5. Although either glyceraldehyde or NADPH could dissociate first, the release of glyceraldehyde occurred more rapidly, and it was the release of NADPH which was rate limiting. At high glycerol concentrations an abortive *E*–NADPH–glycerol complex could form, thus accelerating the dissociation of NADPH. At pH 7.5 the rate limiting step was release of glycerol, and no such activation could occur.

The multivalent anion effect

Sulfate has been reported to have an inhibitory effect upon aldose reductase from some sources (ox brain[6] and *Candida utilis*[8]) while it appeared to activate the enzyme from other sources (lens[4] and Saccharomyces[9]). The present study has found that the effect may be more generally that of multivalent anions. Both sulfate and pyrophosphate inhibited the forward reaction (at pH 7.5) and activated the reverse reaction (pH 9.0). This behavior can be explained on the basis of the mechanisms for the reactions as presented in Fig. 5, assuming that multivalent anions modify the enzyme in such a way that it binds the cofactor less tightly. One possible mechanism for this effect is the following. At pH 7.5, sulfate and pyrophosphate have been shown to be competitive inhibitors of NADPH saturation. It may be proposed that this was due to the presence of a group in the catalytic site of the enzyme which recognized the pyrophosphate anionic portion of the cofactor molecule. Thus, added multivalent anions would have been expected to give competitive inhibition. It was noted in support of this that pyrophosphate itself was a more effective inhibitor than sulfate. According to the mechanism proposed for the reaction at pH 7.5, multivalent anions would effect only the association between cofactor and enzyme: since this did not alter the rate limiting step (release of glycerol), the maximum velocity remained unchanged.

Binding of the cofactor to the enzyme was due partially to recognition of the 2′-phosphate moiety of the cofactor by the catalytic site[12]. If anions were capable of binding to the pyrophosphate recognizing site, and thus modifying the enzyme, the cofactor would still be bound, but less tightly. Therefore, if the dissociation of

the cofactor were the rate limiting step, the maximum velocity as well as the K_m would be increased in the presence of added multivalent anions. This was, in fact, the case, and it is in complete agreement with the mechanism at pH 9.0 for the reverse reaction.

It is interesting to note in this connection that for aldose reductase from placenta and seminal vesicles[2] in the forward reaction both substrate (glyceraldehyde) and sulfate activation were observed, while for the reverse reaction glycerol did not activate and sulfate was inhibitory. Thus, activation by the substrate and multivalent anions occurred together because they were both capable of increasing the velocity of the same (rate limiting) step. To give further support to this conclusion an experiment was performed to determine if glycerol activation would occur with 50 mM $Na_4P_2O_7$ present in all reactions. The results are shown in Fig. 4. The double reciprocal plot became linear indicating that $Na_4P_2O_7$ had already activated the enzyme, and high glycerol concentrations did not give further activation. (The v_{max} was lower when $Na_4P_2O_7$ was added because it was in 400-fold excess to the $NADP^+$ present; it, therefore, reduced the effective concentration of $NADP^+$ to lower than saturating levels.) It was for this reason that only 20 mM glycerol (a subactivating concentration) was used in the anion activation studies (Table I).

The question might be raised: upon continued addition of an anion would not the entire population of enzyme molecules present be modified by binding of the anion and the K_m (app) for $NADP^+$ and the maximum velocity approach a limit? To a certain extent this may be true. However, the situation is complicated by the presence of two anionic portions of the cofactor which are recognized by the catalytic site: the pyrophosphate group and the 2′-phosphate. It is reasonable that at saturating levels of added anions both recognition sites would be occupied and no cofactor would be bound. Thus, no limiting K_m (app) nor v_{max} would be approached.

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