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Purification and Some Properties of a Hepatic NADPH-Dependent Reductase That Specifically Acts on 1,5-Anhydro-D-Fructose

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Glycogen gives rise to 1,5-anhydro-D-fructose (AF), which is then reduced to 1,5-anhydro-D-glucitol (AG) in animal livers. An enzyme that catalyzes NADPH-dependent reduction of AF to AG was isolated and purified to homogeneity from porcine liver. Its apparent molecular mass was about 38 kDa on the basis of SDS-PAGE, and its monomeric dispersion in aqueous solution was indicated by gel filtration on a Superose 12 column. Amino acid sequences were determined for four peptides obtained from the purified enzyme. The resulting sequences covered about 50% of the whole sequence and indicated a remarkable similarity between the enzyme and aldose reductase. The purified enzyme showed molecular activity of 8.7 s^{-1} on the basis of a molecular mass of 38 kDa, and a K_m value of 0.44 mM for AF at the optimum pH of 7.0. It reduced pyridine-3-aldehyde and 2,3-butanedione effectively, acetaldehyde, glucosone, and glucuronic acid poorly and showed no detectable action on glucose, mannose and fructose. It was inactivated by *p*-chloromercuribenzoic acid to a considerable extent, and the inactivation was partially reversed by 2-mercaptoethanol treatment. It was also sparingly inhibited by relatively high concentrations of glucose, glucose-1(6)-phosphate and 1,5-anhydroglucitol. The reverse reaction, *i.e.*, $NADP^+$-dependent AG oxidation, was not observed. The observed catalytic properties and partial amino acid sequences rule out the possibility that the isolated protein is identical with any known reductase.

Key words: 1,5-anhydro-D-fructose, 1,5-anhydro-D-fructose reductase, 1,5-anhydro-D-glucitol, glycogen, glycogenolysis.

1,5-Anhydro-D-glucitol (AG), the 1-deoxy form of glucopyranose, occurs widely in living organisms including bacteria, animals and plants (*1*). We have recently demonstrated that AG originates from glycogen in animal cells through two steps of enzymatic reaction: glucose residues are eliminated from non-reducing terminals of glycogen as 1,5-anhydro-D-fructose (AF), which is subsequently reduced to AG (*2*). These two reactions constitute a third glycogenolytic pathway next to phosphorolysis and hydrolysis. Since the latter product, AG, is metabolically inert (*3*) and the AF-reducing reaction in cultured cells strongly influenced by glucose concentration in the culture medium (*4*), the possibility arises that this third glycogenolytic pathway plays significant roles in control of glycogen metabolism. This possibility will be further evaluated through examination of the properties of the two enzymes involved. In both cultured cells and rat liver lysate, exogenous AF was readily reduced to AG (*4*), while the endogenous AF was produced in only a trace amount (*2*). Therefore, the enzyme responsible for AG production was chosen as the first target of our attempt to isolate the related enzymes. Glycogen is most abundant in the liver, and our previous studies indicated that the liver lysate had the highest AF-reducing activity (*2*). Although the rat liver showed much higher AG-producing activity than the porcine liver in our preliminary studies, we used the latter as the enzyme source in the first purification because of its availability in larger quantity.

MATERIALS AND METHODS

Materials—AG and a clinical AG assay kit (Lana-1,5 AG) were the products of Nippon Kayaku (Tokyo). The assay kit comprised a two-layer column to remove proteins and glucose, and a color-developing solution containing glucose 2-oxidase for AG oxidation, horseradish peroxidase, and 2,2′-azinobis(3-ethylbenzothiazolin-6-sulfonic acid) (*5*). AF was synthesized from AG by oxidization with glucose 2-oxidase (Takara Shuzo) and purified by HPLC on a Shodex SP-0810 column (ϕ8 mm × 300 mm; Showa Denko) according to Kametani *et al.* (*6*). The anion exchange cellulose, DE52 was purchased from Wattman International. Sephacryl S-300 and Red Sepharose CL-6B were from Pharmacia Biotech. Lysyl endopeptidase and all other reagents were from Wako Pure Chemical Industries.

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Abbreviations: AF, 1,5-anhydro-D-fructose; AG, 1,5-anhydro-D-glucitol; PCMB, *p*-chloromercuribenzoic acid.

Enzyme Assay—We used two methods for measurement of the AF-reducing reaction. For the crude enzyme preparations, the reaction was carried out in 100 mM phosphate buffer (pH 7.0, 200 μl in total) containing 0.4 mM NADPH, 0.3 mM AF, and an appropriate amount of an enzyme source. The reaction mixture was incubated at 37°C for 1 h, and the amount of AG produced was determined using the clinical AG assay kit (*5*). For the purified enzyme preparation, the reaction was followed by monitoring the decrease of absorbance at 340 nm due to NADPH consumption; the reaction was carried out in a total volume of 1 ml under otherwise the same conditions as the other method unless otherwise indicated.

One unit of the reductase is here defined as the amount of enzyme which reduces 1 μmol of AF in 1 min under the conditions described above. For evaluation of kinetic properties of the purified enzyme, the initial rate was taken as the average rate determined within the first 5% of the whole reduction.

Protein concentration was determined by the method of Bradford (*7*) using bovine plasma gamma globulin (standard I, Bio-Rad Laboratories) as the standard.

Enzyme Purification—1st step: 500 g of frozen porcine liver was thawed in running water, cut into 1-cm cubes, and thoroughly homogenized in ice-cold 20 mM sodium phosphate buffer (pH 7.0, buffer A) using a domestic blender. Then *ca.* 2% volume of 1 M acetic acid was added to the homogenate to decrease the pH to about 5. This pH yielded a clearer supernatant in the subsequent centrifugation at 25,000 $\times g$ for 20 min. Immediately after separation of the resulting supernatant, 2% volume of 1 M NaOH was added to restore the pH to neutral.

2nd step: The supernatant from the first step was treated with 60% saturation of ammonium sulfate, stirred at 4°C for 1 h, and centrifuged at 25,000 $\times g$ for 20 min. The resulting precipitate was dissolved in a minimum volume (50 ml) of buffer A, and the resulting solution was dialyzed against buffer A overnight. The dialyzed mixture was then applied to a DE-52 column (ϕ 5.0 cm $\times$ 100 cm) which had been equilibrated with buffer A. The column was washed with 2 liters of buffer A, then the reductase activity was eluted with 3 liters of buffer A containing 0.2 M NaCl.

3rd step: The pooled active fractions (2.0 liters in total) from the DE-52 column were treated with 60% saturation of ammonium sulfate and stirred at 4°C for 1 h. The resulting precipitate was collected by centrifugation at 15,000 $\times g$ for 15 min, dissolved in a minimum volume (50 ml) of buffer A, and again centrifuged at 15,000 $\times g$ for 15 min. The clear supernatant was applied on a Sephacryl S-300 column (ϕ 2.6 cm $\times$ 200 cm) which had been equilibrated with 50 mM sodium phosphate buffer (pH 7.0). The reductase activity was eluted at an elution volume of *ca.* 400 ml.

4th step: The enzyme activity was again recovered from the pooled active fractions by precipitation with ammonium sulfate at 60% saturation and the recovered precipitate was dissolved in a minimum volume (10 ml) of buffer A. The resulting solution was dialyzed against buffer A overnight and applied to a DE-52 column (ϕ 2.6 cm $\times$ 40 cm) previously equilibrated with buffer A. The column was first washed with 200 ml of buffer A, then a linear concentration gradient of NaCl from 0 to 0.5 M in 600 ml of buffer A was applied. The reductase activity was eluted at an elution volume of *ca.* 200 ml from the start of the gradient.

5th step: Active fractions in step 4 (50 ml) were applied directly to a Red Sepharose CL-6B column (ϕ 0.9 cm $\times$ 15 cm). The column was washed with 50 ml of 0.5 M NaCl in buffer A, and the enzyme was eluted with 50 ml of 1 M NaCl in buffer A. All the eluate in the last elution was collected.

6th step: The reductase activity was recovered from the collected fraction in the 5th step by ammonium sulfate precipitation at 80% saturation and applied with a minimum volume (0.5 ml) of 50 mM phosphate buffer (pH 7.0) on a Superose 12 column (volume 25 ml), which was pre-equilibrated and driven with the same buffer. The enzyme activity was eluted at 20 ml.

7th step: The active fraction from step 6 was diluted with the same volume of distilled water and applied to a Mono Q column (volume 1 ml) previously equilibrated with buffer A. The enzyme was eluted with a linear concentration gradient of NaCl (0-0.3 M) in 15 ml of buffer A. The major peak of absorption at 280 nm was collected and stored under refrigeration.

Amino Acid Sequencing—The purified enzyme (200 pmol) was incubated with lysyl endopeptidase (25 pmol) overnight at 37°C in 100 μl of 100 mM Tris-HCl buffer (pH 9.0) containing 2 M urea. The resulting full digest was applied directly on a reverse-phase column which was pre-equilibrated with water containing 0.1% trifluoroacetic acid. The column was eluted with a linear gradient of acetonitrile concentration in water (0-80% in 60 min) at a flow rate of 1 ml/min. Several isolated peaks were collected and subjected to peptide sequencing, which was carried out by Applied Biosystems Division, Perkin Elmer Japan.

RESULTS

A New Reductase—The protein responsible for the reductase activity was purified to homogeneity by a combination of chromatographies including three steps of anion exchange, two steps of molecular sieve, and one step of affinity chromatography. Results of purification are summarized in Table I. The AF-reducing activity was separated

TABLE I. **Purification of 1,5-anhydro-D-fructose reductase activity.**

Step	Total protein (mg)	Total activity (unit)	Specific activity (unit/mg protein)	Recovery (%)	Purification (fold)
1. Liver lysate	97,970	223	2.28×10^{-3}	100	1
2. 1st DE 52	33,370	155	4.64×10^{-3}	69.5	2.04
3. Sephacryl S-300	3,263	107	3.28×10^{-2}	48.0	14.4
4. 2nd DE 52	1,011	69.1	6.83×10^{-2}	31.0	30.0
5. Red Sepharose CL-6B	69.7	27.2	3.90×10^{-1}	12.2	171
6. Superose 12	12.8	11.1	8.67×10^{-1}	5.0	380
7. Mono Q	4.4	5.1	1.16	2.3	510

into two distinct fractions in the first anion exchange chromatography: a minor portion (about 20% of the total activity) of the activity was eluted with buffer A, and the major part (about 80% of the total activity) with buffer A containing 0.2 M NaCl. The latter fraction was purified further. In the final two chromatographies on Superose 12 and MonoQ, the reductase activity was associated with single and symmetrical UV peaks. The final peak from the MonoQ column attained 500-fold purification and was perfectly homogeneous as judged by SDS-PAGE. The molecular mass estimated from its mobility on the SDS-PAGE was 38 kDa, and that from the elution volume in molecular sieve chromatography on the Superose 12 column was about 35 kDa.

In each step of purification, the active fractions were stored at 4°C. When these chromatographic fractions were stored frozen at −20°C for 3 days and then thawed, their reductase activities were reduced to about 75%. On the other hand, the purified enzyme was stable at 4°C and storable without discernible loss of the AF-reducing activity at least for one month.

Of the isolated peptides recovered from the lysyl endopeptidase-digest of the purified protein through reverse-phase HPLC, four peptides were large and homogenous enough to determine their amino acid sequences. Homologous sequences to these fragments were sought for by a BLAST search in the data bases GenBank and Swissplot, which selected porcine aldose reductase [EC 1.1.1.21] as the most similar protein among those registered in the two data banks on 17 May 1997. These four partial sequences are, therefore, overlaid on the full sequence of porcine aldose reductase (*8*) in Fig. 1, where the positioning of each fragment is based on its sequence homology with the respective segment of the aldose reductase. The figure shows reasonable assignment of each fragment to a particular segment of the aldose reductase and reveals a striking homology between the purified protein and the aldose reductase.

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  1          kIPVLGLGTWQAAPGGEVTEAVKVAIDTGYRHPDSAYLYHNEDEVGVGIQA
             *.*.******.  * *.********** ****.*.*..*.**.***.*.*
  1 ASHLVLYTGAKMPILGLGTWK-SPPGKVTEAVKVAIDLGYRHIDCAHVYQNENEVGLGLQE

 61 KIDEGVVRREDLFIVSK                    kXXYLDLXNIHWPMQFKPGEV
    *.   **.*********                    * **** **** ****.
 61 KLQGQVVKREDLFIVSKLWCTDHEKNLVKGACQTTLRDLKLDYLDLYLIHWPTGFKPGKD

121 DLPVDRSGMIVASNTDF                              k-XPV
    .*.*.*.*.*....**                                *.
121 PFPLDGDGNVVPDESDFVETWEAMEELVDEGLVKAIGVSNFNHLQVEKILNKPGLKYKPA

181 TNQIESHPYLTQ-K-ISFXQSRNVSVTAYRPLGGS------SEGVPLLEDPVIQTXA
    .**** ******  *.. .*. . ****.***..      .*. .***** *.. *
181 VNQIEVHPYLTQEKLIEYCKSKGIVVTAYSPLGSPDRPWAKPEDPSLLEDPRIKAIAAKY

241                          kRILENFQVFDFELSEQDMTDLLGLDRNLRLSAF
                             ** ********** .**..**. .** *..*.
241 NKTTAQVLIRFPMQRNLIVIPKSVTPERIAENFQVFDFELSPEDMNTLLSYNRNWRVCAL

301 PIAENHXKxypfk
        * * ***
301 MSCASH-KDYPFHEEY
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Fig. 1. **Comparison of the partial amino acid sequences of AF reductase and aldose reductase.** Amino acid sequences of the four peptide fragments determined in the present study are placed over the corresponding segments of porcine aldose reductase to which the fragments were indicated to have the highest similarity by a BLAST search. The corresponding segments were determined in the reported amino acid sequence of the reductase by a Genetyx homology search. • indicates an identical pair and · indicates a homologous pair. Small letters indicate either lysyl residues expected from the specificity of the digestion enzyme used or those in a sequence which is too short for the localization to be decisive.

Catalytic Properties—Optimum pH: The purified enzyme shows the highest activity at neutral pH. Figure 2 shows rate of AF reduction at various pHs in 100 mM sodium acetate buffer (pH=3.6-5.6), 100 mM sodium phosphate buffer (pH=5.7-7.9), or 50 mM Tris-HCl buffer (pH=7.4-9.0) under otherwise the standard conditions. The highest activity was observed at pH 7.0 in the sodium phosphate buffer.

Optimum temperature: The purified enzyme was relatively heat labile (Fig. 3). The enzyme reaction produced the maximum amount of the product at 37°C during 1 h of incubation. At this temperature, the enzyme activity was

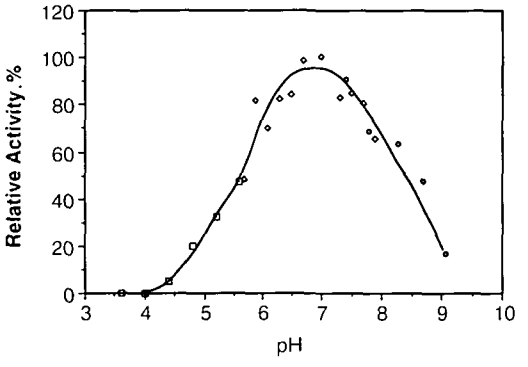

Fig. 2. **Relative activity of AF reductase at various pHs.** The enzyme reaction was carried out either in a 100 mM buffer of acetate-Na (squares) or phosphate-Na (diamonds), or a 50 mM buffer of Tris-HCl (circles) under otherwise the standard conditions. The activities shown on the ordinate are relative to the maximum activity observed at pH 7 in the phosphate buffer.

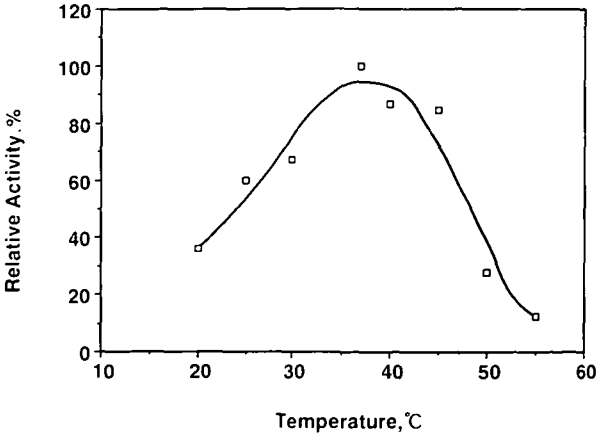

Fig. 3. **Effect of temperature on AG production in 1-h reaction catalyzed by AF reductase.** The enzyme reaction was carried out at various temperatures under otherwise the standard conditions. The rate of AF reduction averaged over the whole reaction time is given on the ordinate.

TABLE II. **Substrate specificity of 1,5-anhydro-D-fructose reductase from porcine liver.**

Substrate	Concentration (mM)	Coenzyme (0.2 mM)	Relative activity (%)
AF	0.3	NADPH	100
AF	0.3	NADH	3.8
Glucose	0.3	NADPH	0
Fructose	0.3	NADPH	0
Mannose	0.3	NADPH	0
Glucosone	0.5	NADPH	2.0
Acetone	0.5	NADPH	0
2,3-Butanedione	0.5	NADPH	53.4
Formaldehyde	0.5	NADPH	7.3
Acetaldehyde	0.5	NADPH	2.7
DL-Glyceraldehyde	0.5	NADPH	0
Pyridine-3-aldehyde	0.5	NADPH	80.5
Glucronic acid	0.5	NADPH	15.2

TABLE III. **Inhibitors of 1,5-anhydro-D-fructose reductase.**

Inhibitor	Final concentration (mM)	Preincubation time (min)	Inhibition (%)
Glucose	100	0	2.6
Glucose-1-phosphate	50	0	25.5
Glucose-6-phosphate	50	0	2.0
AG	100	0	2.3
Ascorbic acid	1.0	0	29.9
Barbital	1.0	0	14.8
Phenobarbital	1.0	0	21.6
EDTA	10.0	10	59.0
Iodoacetic acid	1.0	10	26.8
Iodoacetamide	1.0	10	41.2
PCMB	0.1	10	79.4
PCMB +2-Mercaptoethanol[a]	0.1+1.0	10+10	63.0
2-Mercaptoethanol	1.0	10	2.3

PCMB, *p*-chloromercuribenzoic acid. [a]The enzyme was preincubated with 0.1 mM PCMB for 10 min, then 1.0 mM 2-mercaptoethanol was added and the enzyme was preincubated for another 10 min.

durable for a prolonged period of time in the reaction mixture; at a high concentration of AF (2 mM) the reaction rate was constant for at least 10 min. On the other hand, the enzyme activity decreased with time at 45°C and above; the higher the reaction temperature was, the more rapidly the enzyme activity decayed in the reaction mixture.

Substrate specificity: Table II compares the susceptibilities of various carbonyl compounds to the purified enzyme. All the enzymatic reactions were carried out under the standard conditions except substrate concentration, which is indicated in the table. Among the compounds listed in the table, the purified enzyme reduced AF most efficiently and glucosone only slightly. Pyridine-3-aldehyde and 2,3-butanedione were also good substrates. The enzyme showed practically no activity for glucose and fructose.

Inhibitors: The effects of various compounds on reduction of AF by the purified enzyme are summarized in Table III. Where indicated in the table, the enzyme was first incubated with an inhibitor for 10 min, then the reaction was started by addition of the substrate. High concentrations of AG, glucose and glucose-6-phosphate showed little inhibitory effect, while glucose-1-phosphate exerted a larger effect than other glucose-related compounds tested. Thiol reagents inhibited the enzyme activity to some extent. The inactivation by *p*-chloromercuribenzoic acid (PCMB) was partially reversed by incubation with 2-mercaptoethanol.

Co-enzyme specificity: An almost absolute requirement for NADPH was observed in the AF reduction by the purified enzyme; the rate of reduction in the presence of 0.2 mM NADH was as low as 4% of that in the presence of 0.2 mM NADPH (Table II).

Stoichiometry: Optical determination of NADPH consumption and the enzymatic determination of the concomitantly produced AG in the standard reductase reaction showed that 1.19 mol of NADPH was oxidized per mol of AF reduced.

Kinetics: At a fixed concentration of NADPH (0.4 mM), the AF reduction by the purified enzyme showed a Michaelis-Menten type kinetics; the apparent K_m for AF and the molecular activity were 0.44 mM and 8.7 s^{-1} (molecular mass of the enzyme is assumed to be 38 kDa), respectively, under the standard conditions.

Reverse reaction: No detectable reverse reaction occurred under any conditions examined; no increase of absorbance at 340 nm was observed upon incubation at 37°C under such an extreme composition of the reaction mixture as 2 mM $NADP^+$, 100 mM AG, and 1 μg/ml of purified enzyme in 100 mM sodium phosphate buffer (pH 7.0).

DISCUSSION

The purification of a protein by 500-fold in terms of AF-reducing activity resulted in isolation of a single 38-kDa protein which was homogenous on the basis of SDS-PAGE. Comparison of partial sequences of this protein with sequences in databases indicates that it is most closely related to aldose reductases on the basis of primary structure. The fact that the purified protein shares homologous sequences with NADPH-dependent reductases confirms that it was also a reductase; and because the final preparation was highly homogenous, it is highly probable that the 38-kDa protein is itself responsible for the catalytic reduction of AF by NADPH. On the other hand, the sequence difference from any reductase registered in the databases indicates that the 38-kDa protein is a new type of reductase. It showed higher preference for AF than for other carbonyl compounds (Table II), which are known to be preferred substrates of other NADPH-dependent carbonyl group-reducing enzymes. Accordingly, the 38-kDa protein was designated as an AF reductase.

The reductase activity was inhibited by such SH-reagents as PCMB and alkyliodines. The inhibitory effect of PCMB could be partly reversed by subsequent addition of a mercaptan. These observations indicate that the enzyme involves cysteine residues in its catalytic center, a structural feature that is common to most NAD(P)H-requiring oxidoreductases.

AF reductase activity was inhibited to only a limited extent by high concentrations of glucose and its phosphate esters. This fact stands in a sharp contrast to the previous observation in cultured cells, H-35 and K-562: active reduction of AF in the cells was largely suppressed by the presence of glucose in the medium (*4*). Since the reduction of AF to AG is a single and irreversible enzymatic reaction, this apparent disagreement suggests the involvement of an unknown effector whose concentration in the cells is influenced by glucose concentration in the medium. Elucidation

of the mechanism by which AF reductase activity is controlled *in vivo* may clarify the physiological significance of AF reductase in the cells.

The present purification indicated that AF reductase constituted 0.2% of proteins in the liver homogenate. This abundance is seemingly too large for very low content of AF in animal livers. We have previously demonstrated, however, that the cultured cells, H-35 and K-562, have high capacities to reduce AF in the medium (*4*); and this observation is compatible with the relatively high abundance of AF reductase indicated by the present purification study. AF originates from glycogen and is converted to AG in the cell. Since glycogen occurs in almost all cells, AF may also arise in most cells. Plasma membranes appear to be much less permeable to AF than to AG (*4*), and thus AF is much more likely to be retained within the cells than AG. Accordingly, AF reductase is considered to be mainly responsible for AF clearance from the cells. A high abundance of AF reductase indicates that the cells have a high capacity for AF clearance; and this accords well with our observation that the natural abundance of AF is generally very low (*2*). Molecules or molecular states involved in biological signaling systems are generally of low abundance and short-lived. This character of signaling molecules suggests that AF may be an effector in the control system of glycogen metabolism; and this possibility is now under examination in our laboratory.

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