BIOORGANIC CHEMISTRY **26,** 233–243 (1998)
ARTICLE NO. BH981104

Binding of Inhibiting Adducts of Ketones and NAD^+ to Alcohol Dehydrogenase from *Drosophila melanogaster*

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Received May 7, 1998

Drosophila alcohol dehydrogenase (DADH) can be converted with NAD^+ and ketone into more negatively charged isoforms. Completely modified ADH isoforms are inactive, but activity is regained after native polyacrylamide gel electrophoresis depending on the ketone used. When unmodified ADH is incubated with NAD^+ and acetylacetone, more negatively charged bands appear. Modified ADH isoforms bind to a Mono-Q column, while unmodified ADH and NAD^+ do not. A covalently bound adduct is also formed between NAD^+ and ketone in the absence of enzyme. These adducts can be purified by ion-exchange FPLC and have been characterized by mass spectrometry and UV spectroscopy and also form inactive isoforms after incubation with unmodified enzyme. The effect of temperature increase on unmodified and modified *Drosophila melanogaster* variants was determined by circular dichroism experiments. Unfolding temperatures (T_m) of the modified ADH isoforms were 5 to 20° higher than those of the unmodified ones, indicating that the enzyme molecule becomes more compact upon adduct binding.

Key Words: *Drosophila melanogaster*; alcohol dehydrogenase; adduct binding; stability; denaturation.

INTRODUCTION

Drosophila alcohol dehydrogenase (ADH)[2] belongs to the family of short-chain dehydrogenases/reductases (*1*, *2*). Enzymes belonging to this family generally convert substrates with large, complex chemical structures. The major function of the enzyme from *Drosophila* is to convert and detoxify ethanol, a ubiquitous compound in the environment of these insects (*3*, *4*). However, the enzyme has higher specific activities on secondary alcohols than on primary ones. Elsewhere, we present kinetic data on the conversion of secondary alcohols by *Drosophila simulans* (*5*), a sibling species of the well-known *Drosophila melanogaster*, and demonstrate the effect of substrate activation, a phenomenon caused by formation of abortive ternary complexes of enzyme with NADH and secondary alcohols, from which NADH dissociates faster than from binary complexes of NADH with enzyme (the rate-limiting step in the reaction with secondary alcohols).

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[2] Abbreviations used: ADH, alcohol dehydrogenase (EC 1.1.1.1); ADH-sim, *Drosophila simulans* alcohol dehydrogenase; NAD^+, nicotinamide adenine dinucleotide; NADH, reduced NAD^+; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide; PMS, phenazine methosulfate.

0045-2068/98 $25.00

Drosophila alcohol dehydrogenase also forms abortive ternary complexes with NAD^+ and ketone, followed by the formation of a rather stable covalent adduct between NAD^+ and ketone leading to strong enzyme inhibitors. These latter ternary complexes are very stable and their formation leads to enzyme inhibition. They can be recognized from electrophoretic patterns, using native polyacrylamide gel electrophoresis, by the presence of more negatively charged isoforms of ADH with two NAD-carbonyl compounds per dimer (ADH-1) and one per dimer (ADH-3), respectively, in addition to the unmodified enzyme (ADH-5).

The formation of these isoforms has been observed *in vitro*, e.g., by incubation of the enzyme with NAD^+ and acetone (*6–11*), or *in vivo* by additions to the food, such as acetone and isopropanol (*12*, *13*). Although modified *Drosophila* ADH in solutions is an irreversibly inactivated enzyme, slow reactivation occurs on native gels on activity staining with a strong electron acceptor. But reactivation does not happen *in vivo* (*8*, *9*, *14–16*).

The nature of the adduct responsible for the formation of a ternary dead-end complex was discussed by Winberg *et al.* (*16*) and Schwartz *et al.* (*17*). However, several molecular characteristics remain unexplained. In this paper we demonstrate the formation of different types of adducts, depending on the ketones used, which also differ in their capability to be reactivated on native polyacrylamide gels. We could also demonstrate that ADH variants differing in thermostability are stabilized, by adduct formation, to isoforms with identical unfolding temperature.

MATERIALS AND METHODS

Materials

NAD^+ and Tris were purchased from Boehringer (Mannheim, Germany). Acrylamide, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and Coomassie brilliant blue G-250 were from Serva Feinbiochemica (Heidelberg, Germany). Phenazine methosulfate (PMS) and bovine serum albumin (BSA) were from Sigma Chemical Co. (St. Louis, MO). Sodium dodecyl sulfate (SDS), ammonium persulfate (APS), veronal, and *N′*,*N′*-methylene-bis-acrylamide were obtained from BDH Chemicals (Poole, UK). Acetic acid was from Janssen Chimica (Tilburg, The Netherlands). Glycine, 2-butanone, 2-pentanone, 2-hexanone, 2-heptanone, 2-octanone, 3-pentanone, 3-hexanone, 3-heptanone, 3-octanone, and acetylacetone were from Fluka Biochemika (Buchs, Switzerland). All other reagents and biochemicals were from Merck (Darmstadt, Germany).

Purified alcohol dehydrogenases of two *D. melanogaster* variants, ADH-71k, a thermostable one (*18–20*), and ADH-S, respectively, were used in most experiments. The enzymes were purified according to Smilda *et al.* (*21*).

Conversion of Drosophila ADH and Adduct Formation and Purification

To convert unmodified alcohol dehydrogenase into the modified ADH isoform, equal amounts of enzyme preparations of *Drosophila* ADH were incubated in the presence of 18 mM NAD^+ and 240 mM ketone, in 20 mM Tris/HCl buffer, pH 8.6.

Incubation was performed for at least one night at room temperature in the dark, but an incubation time of 1 h was usually enough (results not shown). The modified isoform was separated from unbound NAD^+ and ketone by gel filtration FPLC on a Superose 12 column and a Superdex 75 column connected in series (Pharmacia, Uppsala, Sweden), using 20 mM Tris/HCl buffer, pH 8.6. Modified *Drosophila* ADH was purified by ion-exchange FPLC on a Mono-Q column (Pharmacia) using 20 mM Tris/HCl buffer, pH 8.6, and a linear gradient of 0–1 M NaCl.

Adducts were formed between NAD^+ and ketone by incubation of 18 mM NAD^+ with 240 mM ketone in 20 mM Tris/HCl buffer, pH 8.6. The adducts were purified by ion-exchange FPLC on a Mono-Q column (Pharmacia) using 20 mM Tris/HCl buffer, pH 8.6, and a linear gradient of 0–1 M NaCl. The molecular masses of adducts were determined by matrix-assisted laser-desorption ionization time-of-flight mass spectrometry with a TOFSPEC E mass spectrometer (Micromass, Manchester, UK) using 2,5-dihydroxybenzoic acid (BDH) as matrix. Absorption spectra were recorded with a Kontron Uvikon 930 UV/VIS double-beam spectrophotometer at 25°C.

Denaturation

Circular dichroism (CD) spectroscopy was carried out using an AVIV Model 62DS circular dichroism spectrometer (Aviv, Lakewood). Purified samples of unmodified *D. melanogaster* ADH-S and ADH-71k or complexes of ADH-S and ADH-71k with NAD^+ and ketone were analyzed in 5 mM sodium phosphate buffer, pH 7.5, by measurement of the ellipticity at 220 nm at 25°C, at increasing temperatures from 25 to 95°C. The data were collected in a 1-mm path length cuvette. Ellipticities are expressed in units of degree per square centimeter per decimole, using the mean residue concentration.

Modified alcohol dehydrogenase with NAD^+ and ketone was also digested with trypsin (2%, w/w) in 20 mM Tris/HCl, pH 8.6, at 37°C for 4 h followed by reversed-phase HPLC with a 0–67% gradient of acetonitrile in 0.1% trifluoroacetic acid.

Analytical Methods

Native polyacrylamide gel electrophoresis was performed according to the method of Van Delden *et al.* (*22*). Polyacrylamide gels (6%) were prepared in slabgel molds. The gel and electrode buffer used contained 0.041 M veronal/HCl, pH 8.4, equally mixed with 0.15 M Tris/acetate, pH 8.4. Enzyme preparations were used and 10 μl was loaded onto the gel. Electrophoresis was carried out horizontally at 4°C for 4 h with a constant current of 80 mA (24 $\times$ 15.5 cm gels), or at 4°C for 2 h with a constant current of 20 mA (7 $\times$ 6.5 cm gels). After electrophoresis the gels were stained for ADH activity. The staining solution contained 43 mM 2-butanol, 4.5 mM NAD^+, 3.6 mM MTT, and 33 nM PMS in phosphate–buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 6.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.2). Protein concentrations were determined by amino acid analysis after vapor phase on a Hewlett Packard Amino Quant 1090M.

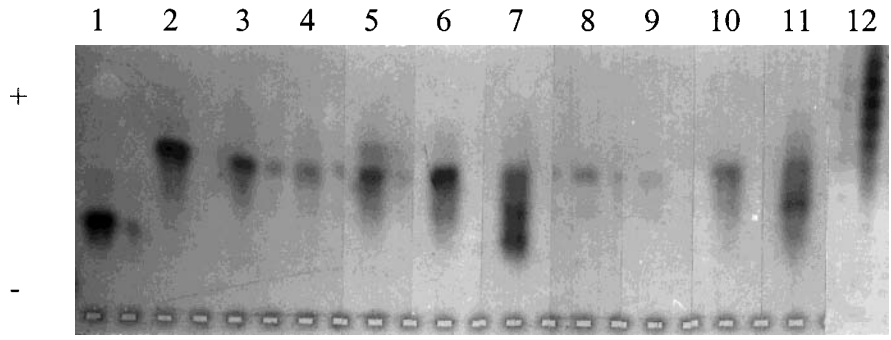

FIG. 1. Native polyacrylamide gel of unmodified *Drosophila melanogaster* ADH and modified ADH isoforms. Ketones used are (2) acetone, (3) 2-butanone, (4) 2-pentanone, (5) 2-hexanone, (6) 2-heptanone, (7) 2-octanone, (8) 3-pentanone, (9) 3-hexanone, (10) 3-heptanone, (11) 3-octanone, and (12) acetylacetone.

RESULTS AND DISCUSSION

Isolation of ADH–NAD–Ketone Complexes and NAD–Ketone Adducts

The conversion of unmodified *D. melanogaster* ADH-71k with several ketones is shown in Fig. 1. After electrophoresis at least one band was found, indicating complete conversion of the unmodified ADH to modified ADH isoforms, while in incompletely converted samples a band with intermediate mobility was also visible. The completely converted isoforms are ADH dimers with one negatively charged NAD–ketone adduct bound per monomer, while in the partially converted isoform only one NAD–ketone adduct is present per dimer. The difference in mobility between completely and partially converted ADH isoforms, and between partially converted ADH and unmodified ADH, is identical to that between homozygous alleloenzymes like *D. melanogaster* ADH-F and ADH-S (*23*). Because the latter differ in one charged residue per subunit (or two per dimer), the binding of each NAD–ketone adduct introduces two additional negative charges in the isoforms.

With most of the ketones investigated only one band was observed. However, in the case of 2-heptanone, 2-octanone, and 3-octanone there is incomplete conversion, because these ketones have a lower affinity to the enzyme. This is in agreement with kinetic studies which showed that the substrate-binding cleft of *Drosophila* ADH has no room for alkyl chains of more than 3–4 carbon atoms (*5*).

Although ternary complexes of *Drosophila* ADH with NAD^+ and ketones are enzymatically inactive in solution, the method used to demonstrate activity in gels after native polyacrylamide gel electrophoresis shows activity in most investigated cases. Probably the NAD-ketone adduct dissociates from the enzyme under these conditions. Only after complete conversion with 3-pentanone, 3-hexanone, 3-heptanone, and 3-octanone little reactivation occurred. This may be explained by an ethyl group having a higher affinity than a methyl group, probably in both the smaller and the larger binding pockets of the enzyme (*5*). The effect of this is that also on the activity-stained gels no dissociation occurs any more.

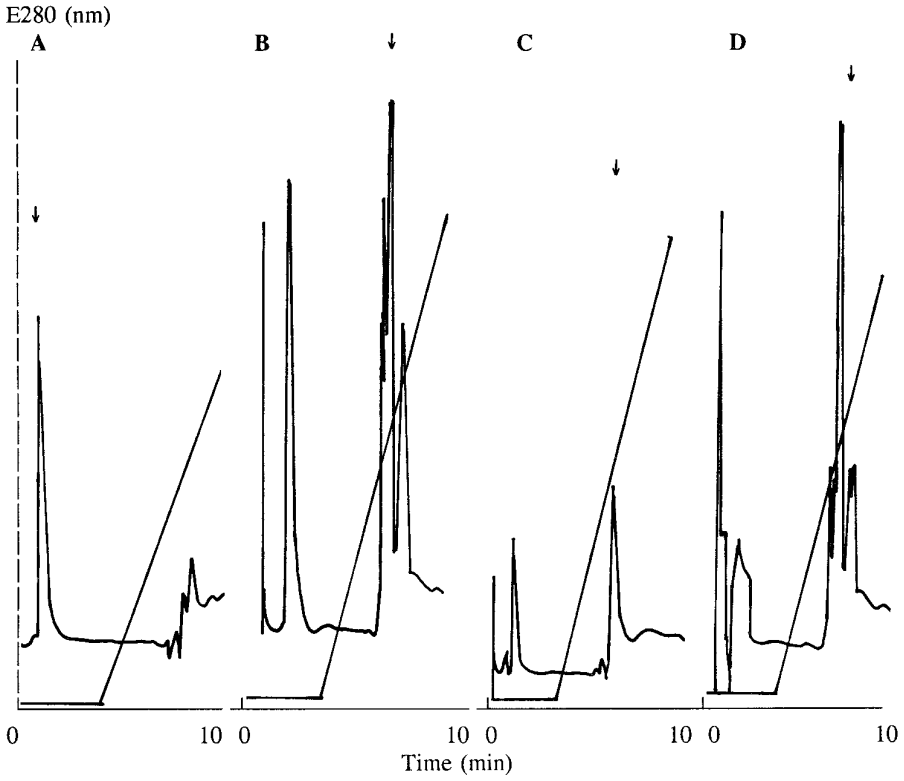

FIG. 2. Elution patterns of unmodified *Drosophila melanogaster* ADH (A) and modified forms with NAD^+ and acetone (B), 3-pentanone (C), and acetylacetone (D), respectively, after ion-exchange chromatography by FPLC on a Mono-Q column at pH 8.6 and elution with a linear gradient (0–1.0 M) NaCl. Peaks were analyzed for the presence of protein by SDS–PAGE. Protein-containing peaks are indicated with an arrow.

Incubation of *Drosophila* ADH with NAD^+ and acetylacetone is exceptional in leading to the formation of about six isoforms, which are more negatively charged than those obtained with the other ketones (Fig. 1). The distances between them indicate that they differ stepwise in charge with one unit per *Drosophila* ADH dimer. The most intensive band represents an isoform which has four more charges per dimer than the completely converted isoforms with the other ketones.

Unmodified *D. melanogaster* alcohol dehydrogenase, when applied on a Mono-Q column, does not bind under the conditions used in contrast to the modified *Drosophila* ADH forms which had to be eluted using an NaCl gradient. The elution patterns of unmodified *Drosophila* ADH and modified ADH with NAD^+ and acetone, 3-pentanone, and acetylacetone, respectively, are shown in Fig. 2. The isoforms with acetone and 3-pentanone elute at the same position, but that with acetylacetone elutes somewhat later, which also indicates that it is more negatively charged than the others.