ACTIVITY OF HORSE LIVER ALCOHOL DEHYDROGENASE SS WITH NADP(H) AS COENZYME AND ITS SENSITIVITY TO BARBITURATES

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Summary. Alcohol dehydrogenase SS, free from other isoenzymes, has been purified from horse livers. The enzyme has high activity with NADP(H) as coenzyme. With NADPH its activity is 3 times more than with NADH. While its affinity for NADPH is less than for NADH, in comparison with the classical ADH its affinity for NADP(H) is increased. In its activity with NADP(H) and inhibition with barbiturates, ADH SS resembles aldehyde reductases.

Alcohol dehydrogenase (ADH) in horse livers occurs in nine, readily identifiable, multiple molecular forms (1). Three of these consist of catalytically (1) and genetically (2) distinct subunits, E and S, and are dimers of the following structures: EE, ES and SS. The solubility characteristics of ADH SS and ADH EE are distinct; while ADH SS is very soluble, ADH EE is readily crystallizable and preferentially isolated from horse livers. Commercial and individual preparations of horse liver ADH, employing the methods of Bonnichsen and Wassen (3), with or without the Dalziel (4) modification, consist of 60-80% of ADH EE and are not representative of the ADH present in horse livers. Activity with 3β-hydroxy-steroids and the corresponding 3-ketones, in addition to activity with the classical ADH substrates, distinguishes ADH SS from ADH EE, the latter inactive towards steroids. Recently, isolation of an enzyme from mammalian brain (5), called aldehyde reductase, linked with NADPH, has been reported. Aldehyde reductases are also present in other tissues (6); some are NADPH specific and inactive with short chain aldehydes, others have dual nucleotide specificity. The designation as aldehyde reductase rather than as alcohol dehydrogenase arises from the difficulty of demonstrating the reverse reaction with NADP. Another characteristic of aldehyde reductases, not exhibited by horse liver ADH, is inhibition by barbiturates and decreased sensitivity to pyrazole (7).

Dalziel and Dickinson (8) have reported that the low activity of the classical

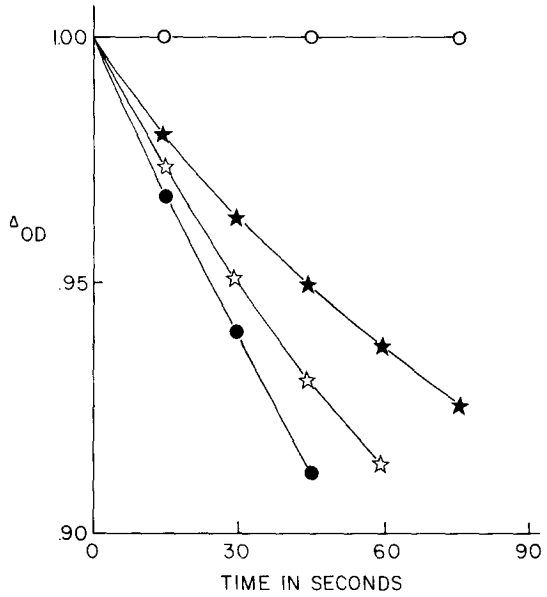

Figure 1: Effect of NAD on the activity of ADH SS with NADH and NADPH. The assay system contained; phosphate buffer 0.1M, pH 7.0; 5βDHT, 114μM; ●—● NADPH, 170μM; O—O NADPH, 170μM + 1.8mM NAD: ☆—☆ NADH, 170μM; ★—★ NADH, 170uM + 1.8mM NAD. 5βDHT was added in 10ul of dioxane. The measurements were made in a Beckman DB-GT spectrophotometer at 25° and 340nm in 3 ml final volume.

ADH with NADP(H) is due to its low affinity for NADP(H) combined with the fact that commercial preparations of NADP contain NAD which functions as an effective competitive inhibitor. The results presented here show that NADP(H) is a better coenzyme for ADH SS than for the classical ADH and that the activity with both NADH and NADPH is inhibited by barbiturates.

ADH SS was prepared from horse livers by ammonium sulphate fractionation followed by chromatography on DEAE-cellulose. The preparation was pure with respect to known ADH isoenzymes, but only 10-17% pure as measured by active site titration with NADH in the presence of isobutyramide (9) or NAD in the presence of pyrazole (10); total protein was determined by the Lowry procedure. The preparation did not oxidize NADH or NADPH, nor did it reduce NAD or NADP in the absence of substrate or have any aldehyde dehydrogenase activity.

Employing a steroidal ketone, 5β-dihydrotestosterone (5βDHT), as substrate, the rate of oxidation of NADH and NADPH(Sigma, USA) by ADH SS was measured and

Table I

Comparison of activity of ADH SS and Boehringer ADH with NADH and NADPH

enzyme	substrate	$\frac{\text{rate with NADPH}}{\text{rate with NADH}}$
ADH SS	5βDHT	1.40
Boehringer ADH (80% EE)	cyclohexanone	0.035

The assay system contained phosphate buffer 0.1M pH 7.0; NADPH or NADH, 170μM; 5βDHT, 114 μM, or cyclohexanone, 14mM; 5βDHT was added in 10μl of dioxane to 3 ml final volume of the assay. The measurements were made in a Beckman DB-GT spectrophotometer at 340nM at 25°.

compared with that of a horse liver ADH preparation (Boehringer und Soehne, Germany) consisting of 80% ADH EE. The results (Table I) show that at equimolar concentrations of nucoleotides the rate of reduction of 5βDHT by NADPH in the presence of ADH SS is greater than the rate with NADH, while under the same conditions the rate of reduction of cyclohexanone by Boehringer ADH and NADPH is only 1/30 of the rate with NADH.

The reduction of 5βDHT by ADH SS and NADPH is inhibited by classical ADH inhibitors such as 1,10-phenanthroline, isobutyramide or pyrazole, indicating that ADH SS itself and not a contaminating non-ADH enzyme is responsible for NADPH-5βDHT activity. With pyrazole and NAD, the Ki value is similar to that reported (10) for the classical ADH (0.1μM); the Ki values for isobutyramide (10mM with either coenzyme), however, are considerably more than for the classical ADH [9μM(11)], indicating that ADH SS is also different from the classical ADH in some of its inhibitor interaction properties. The loss of activity on heat treatment, found to be the same when measured with either NADH or NADPH, shows that the binding sites for both coenzymes are on the same protein molecule. In the presence of NAD (a product of NADH and an alternate product for NADPH), the

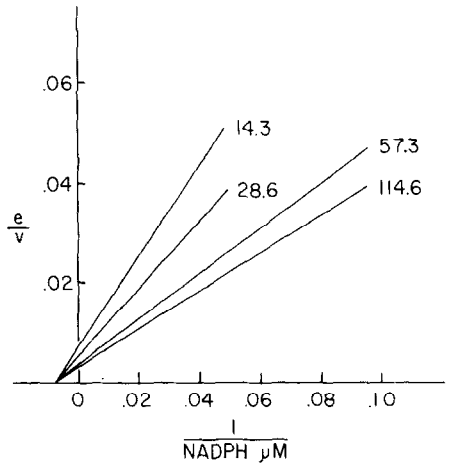

Figure 2: Variation of the reciprocal of the initial rate of NADPH oxidation at four different concentrations of 5βDHT (shown on graph in μM units). The assay was done in phosphate buffer, 0.1M pH 7.0 at 25 in a Beckman DB-GT spectrophotometer at 340nm. 5βDHT was added in 10μl of dioxane to the final volume of 3 ml. e - concentration of enzyme active sites in μN units; v - rate of NADPH oxidation in μM/min.

initial rate of reduction of 5βDHT with either NADH or NADPH is inhibited, the inhibition of activity with NADPH being considerably more (Fig.1). In the presence of equimolar concentrations (170μM) of NADH and NADPH the initial rate of reduction of 5βDHT is not additive but equal to the rate of reduction with NADH alone. These results suggest that the affinity of ADH SS for NADPH is less than for NADH and demonstrate that the binding sites for both coenzymes are the same.

In Fig. 2 the Lineweaver-Burk (12) plots for ADH SS are shown at varied NADPH concentrations and four concentrations of 5βDHT. The slope effect and the intercept effect in the primary (13) plot pattern indicates a sequential mechanism (13). The Lineweaver-Burk plots (Fig.2) intersect at an X coordinate independently of whether NADPH or 5βDHT (experiments with constant NADPH and varied 5βDHT are not shown here but were carried out) is used as a variable substrate. Thus the Km for NADPH is independent of the concentration of 5βDHT and the Km for 5βDHT is independent of the concentration of the nucleotide; the Km values represent dissociation constants of ADH SS with these substrates. Consequently, even if the mechanism of the reaction is sequential it could not possibly be of the

Table II

Effect of barbiturates on the reduction of 5βDHT by ADH SS and acetaldehyde by Boehringer ADH.

Inhibitor conc. (mM)	enzyme activity as % control		
	ADH SS		Boehringer ADH
	NADH	NADPH	NADH
phenobarbital			
0.0	100	100	100
1.0	68	69	100
5.0	54	55	90
pentobarbital			
0.0	100	100	100
1.0	66	67	100
2.0	-	-	99
5.0	-	-	89

The assay system contained: phosphate buffer 0.1M, pH 7.0; NADH or NADPH 170μM; ADH SS and Boehringer ADH were preincubated with inhibitors and nucleotides for 3 min. before the reaction was started by addition of 5βDHT (final concentration 57μM, added in 10μl of dioxane) or acetaldehyde (1.2mM). The results are means of triplicate determinations carried out at 25° in a Beckman DB-GT spectrophotometer at 340nm at 25°.

ordered type established by Theorell and Chance (14). Since the constants are of similar magnitude (30μM for 5βDHT and 140μM for NADPH) a random mechanism is suggested. Waller, Theorell and Sjovall (15) using 3β-hydroxy-5β-cholanic acid and NAD also concluded that the mechanism of steroid dehydrogenation by horse liver ADH is distinct from that of Theorell and Chance (14). The calculated Km value for NADPH at saturating concentrations of 5βDHT for ADH SS is 25 times less than reported for the classical preparations of horse liver ADH. The active site of ADH SS is, therefore, distinct, not only with respect to substrate specificity, but also with respect to interaction with nucleotides; the dissociation constant for the binary NADPH - enzyme complex is considerably less: 140μM for ADH SS and 3750μM(8) for the classical ADH. The Km value for NADH at pH 7.0 for the classical preparations of horse liver ADH is 10μM(16), and the dissociation constant

under the same conditions, 0.3μM. While the dissociation constant of ADH SS with NADH, determined directly (17), is 0.09μM, our results indicate that the Km value is considerably less than that reported for the classical ADH. The rate of oxidation of NADH with 5βDHT is the same at 1.0μM as at 170μM; the Km value for NADH must be less than 1.0μM and close in value to the dissociation constant. When the dissociation constant for NADH [0.09μM(18)] and NADPH (140μM) are compared it is apparent that NADPH is bound less tightly to ADH SS. It is of interest to note that the turnover number at infinitely saturating concentrations of both NADPH and 5βDHT (8.3 x active site^{-1} x sec^{-1}) is three times more than that at infinitely saturating concentrations of NADH and 5βDHT (2.5 x active site^{-1} x sec^{-1}).

Using NADP (Boehringer und Soehne, Germany) and 5β-androstane-3β-ol-17-one (Sigma, USA) as substrate, only negligible activity could be demonstrated. However, after purification of NADP on DEAE cellulose (8), the activity with NADP approached that with NAD. In 0.1M phosphate buffer at pH 7.0, the Km for NADP was found to be 420μM, as compared to 2100μM for classical horse liver ADH(8). It appears that with NADP and NADPH the affinity of the enzyme has increased in the S modification of the ADH subunit.

In Table II the effect of phenobarbital and pentobarbital on the activity of ADH SS and Boehringer ADH is compared. At 1mM phenobarbital and pentobarbital inhibit 5βDHT reduction by ADH SS 30% with either coenzyme, while the cyclohexanone reduction by Boehringer ADH remains unaffected. At 5mM phenobarbital, however, ADH SS activity is inhibited 45%, while that of Boehringer ADH now also shows a 10% inhibition. Inhibition with barbiturates is thus an inherent property of ADH, more pronounced in the S subunit of the enzyme than in the E subunit.

Aldehyde reductases (5,6,7) comprise a group of enzymes of variable nucleotide specificity, in some cases restricted to NADPH and substrate specificity in some cases also more restricted. Those with dual nucleotide specificity (6), with NADPH as coenzyme, have Km values (26,30 and 44μM) which are two orders of magnitude less than the Km for the classical ADH but only 3 to 6 times less than for

ADH SS. They differ from the classical ADH in their susceptibility to certain inhibitors, and like ADH SS are sensitive to barbiturates. Since there is no doubt (2) that ADH SS is a genetic variant of horse liver ADH the results presented in this paper suggest that the established differences between aldehyde reductases and the classical ADH may not be sufficient to adopt a distinct nomenclature.

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