Substrate Specificity of Citrus Alcohol:NAD Oxidoreductase

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An alcohol:NAD oxidoreductase [EC #1.1.1.1] was extracted from Valencia oranges and examined for substrate specificity with aliphatic alcohols and aldehydes reported in citrus. The oxidoreductase oxidizes alcohols to the corresponding aldehydes in the homologous series from ethanol to octanol. The enzyme reduces aldehydes to the corresponding alcohol in the series from acetaldehyde to decanal. The maximum rates of reduction of the aldehydes are higher than the oxidation rates for the alcohols. The unsaturated alcohols, 2-propen-1-ol, 2-buten-1-ol, 4-penten-1-ol, and 2-hexen-1-ol are oxidized faster than the saturated homologs. The enzyme has a higher affinity for the aldehydes than for the saturated alcohols. Activity of the alcohol:NAD oxidoreductase probably determines the prevalent form of these alcohol-aldehyde pairs in the fruit.

The essence of orange juice contains more than 50 oxygenated compounds, including ten saturated and unsaturated alcohols with their corresponding aldehydes or ketones (Wolford and Attaway, 1967). The characteristic orange juice flavor and aroma depends on the concentration balance between these compounds.

The balance between aliphatic alcohols and carbonyls in citrus is probably maintained by an enzyme similar to the alcohol:NAD oxidoreductase [EC #1.1.1.1] reported in the pea (Eriksson, 1968). The pea enzyme catalyzes the equilibrium reaction between the ethanol–ethanal pair and a large number of higher molecular weight pairs.

Orange juice vesicles contain an alcohol:NAD oxidoreductase that catalyzes the equilibrium reaction between ethanol and ethanal (Bruemmer and Roe, 1970a). If this oxidoreductase can oxidize alcohols and reduce aldehydes that are found in orange juice, it probably functions in the equilibrium reaction between the alcohols and aldehydes in the intact fruit.

The purpose of this study was to determine substrate specificity of citrus alcohol:NAD oxidoreductase and measure relative affinities of the alcohol–aldehyde pairs for the oxidoreductase.

MATERIALS AND METHODS

Mature oranges (*Citrus sinensis*, var. Valencia) were obtained from a local grove. Alcohols and aldehydes were obtained from Pfaltz and Bauer, Inc., Flushing, N.Y., yeast alcohol:NAD oxidoreductase from Boehringer Mannheim Co., New York, N.Y., Polyclar AT from GAF Corp., New York, N.Y., and dithiothreitol, nicotinamide adenine dinucleotide (NAD), and the reduced form (NADH) from Calbiochem Co., Los Angeles, Calif.

Enzyme Extraction. Peeled oranges were frozen in liquid-N_2 and the juice vesicles separated from seeds and coarse section membranes. Fifty grams of vesicles were pulverized in a micromill at $-196°$ C with a calculated amount of 1.0 *M* Tris to raise the pH of the thawed extract to 7.0. The neutralized powder was thawed after mixing with 10 g of presoaked Polyclar AT (25% Polyclar AT in 0.1 *M* potassium phosphate buffer, pH 7.0, containing 5×10^{-3} *M* dithiothreitol) and strained through four layers of cheesecloth The cold extract was clarified by centrifuging at 5000 × *g* for 10 min.

Enzyme Fractionation. The proteins in the extract were fractionated by ammonium sulfate precipitation. Fractions corresponding to 0–35, 35–45, 45–55, 55–65, and 65–90% saturation were prepared and dialyzed for 2 hr against 0.001 *M* Tris, pH 7.0. The dialyzates were clarified by centrifugation at 10,000 × *g* for 10 min before assaying. Protein in the fractions was estimated by the sulfosalicylic acid method of Layne (1957).

Oxidation of Alcohols. The rate of alcohol oxidation was measured spectrophotometrically by recording the initial rate of NAD reduction at 340 nm. (Molar extinction coefficient of reduced NAD = 6.2×10^3.) Reference and sample cuvettes (1 cm light path) contained 0.010 or 0.020 ml of the alcohol in 1.5. ml of 10% Tween 80 in 0.2 *M* Tris buffer, pH 9.0, 0.1 ml of 0.015 *M* NAD, and HOH to 3.0 ml. The reaction was initiated by adding 0.01 ml enzyme preparation (0.5 mg protein/ml) to the sample cuvette. NAD was not reduced by ethanol in absence of enzyme, nor by enzyme in absence of ethanol, nor by heat-treated enzyme in presence of ethanol. NADP could not replace NAD in the assay. Tween 80 was included to disperse the alcohols. At 5% final concentration, it did not affect ethanol oxidation rate.

Reduction of Aldehydes. The rate of aldehyde reduction was measured spectrophotometrically by recording the initial rate of NADH oxidation at 340 nm. Reference and sample cuvettes contained 0.01 or 0.02 ml of the aldehyde in 1.5 ml of 10% Tween 80 in 0.2 *M* potassium phosphate buffer, pH 6.5, 0.02 ml of 0.025 *M* NADH, and HOH to 3.0 ml. The reaction was initiated by adding 0.01 ml enzyme preparation to the sample cuvette. NADH was not oxidized by ethanal in absence of enzyme, nor by enzyme in absence of ethanal, nor by heat-treated enzyme in presence of ethanal. NADPH could not replace NADH in the assay. Tween 80 did not effect ethanal reduction rate.

Identification of Reaction Products. The products of hexanol oxidation and hexanal reduction were identified by gas chromatography. Two milliliters of enzyme preparation was incubated 60 min, 30° C with each of the following systems: (A) 0.012 ml, hexan-1-ol, 0.1 ml of 0.1 *M* NAD, and 1.0 ml 0.1 *M* Tris buffer, pH 9.0; (B) 0.12 ml hexanal, 0.1 ml of 0.1 *M* NADH and 1.0 ml 0.1 *M* potassium phosphate buffer, pH 6.5. After acidification with 0.5 ml 1 *N* HCl, the reaction mixtures were extracted with 15 ml ether. The ether extracts were dehydrated with 100 mg Na_2SO_4 and evaporated to 0.5 ml under N_2. Ten microliter portions of the ether extracts were injected into a Perkin-Elmer Model 154 Vapor Fractometer fitted with a 10-ft × 1/4-in. aluminum column containing 6% diethylglycol succinate on acid washed Chromo-

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sorb W 60/80 *M* at 100° C. The helium gas flow was kept at 70 ml/min. In this gc system, hexanal had a retention time of 3.3 min, and hexanol had a retention time of 6.5 min.

RESULTS

About 95% of the alcohol:NAD oxidoreductase activity of orange juice vesicles precipitated between 45–55% and 55–65% ammonium sulfate saturation. The specific activities of these two fractions with ethanol were 6 and 4 μmoles of NAD reduced per min per mg protein.

Oxidation of Alcohols. Results of assay of the series of alcohols from ethanol to decan-1-ol in the citrus alcohol:NAD oxidoreductase system are shown in Table I. Activity drops off sharply with the higher molecular weight alcohols. Solubility of heptan-1-ol, octan-1-ol, nonan-1-ol, and decan-1-ol limited their concentration to less than half the concentration of other substrates. Only a few of the alcohols are active with the yeast enzyme. Most of these alcohols listed in Table I were found in orange fruit (Nursten and Williams, 1967). The product of the citrus NAD-oxidoreductase reaction with hexan-1-ol was identified by gc as hexanal.

Reduction of Aldehydes. Results of assay of the series of aldehydes from ethanal to decanal in the citrus alcohol:NAD oxidoreductase system are shown in Table II. Activity is relatively high for ethanal to hexanal, but activity decreases rapidly as the series extends from heptanal to decanal. The yeast enzyme does not show the same broad substrate specificity. The product of citrus NADH-oxidoreductase reaction with hexanal was identified by gc as hexan-1-ol.

Affinity for Alcohols and Aldehydes. The Michaelis Constants (K_m) of some alcohols and aldehydes in the citrus alcohol:NAD oxidoreductase system are listed in Table III. These substrates gave typical saturation curves for the enzyme. As the molecular weight of the aldehyde increases, the K_{mr} also increases. These relationships are expressed graphically in Figure 1, where affinity of the enzyme for the substrates (reciprocal of K_m) is plotted against the number of carbon atoms in the saturated and α-β unsaturated alcohols and aldehydes. The enzyme has a 5- to 30-fold greater affinity for aldehydes than for saturated alcohols. The affinities for the 3 and 6 carbon unsaturated alcohols and aldehydes are similar.

Equilibrium Constants (K_{app}). Apparent equilibrium constants (K_{app}) for the partial reaction, alcohol + NAD = aldehyde + NADH were calculated from V_f, V_r, and the Michaelis constants for alcohol (K_{mf}), aldehyde (K_{mr}), NAD ($K_{m'f}$) and NADH ($K_{m'r}$) according to the relationship: $K_{app} = (V_f/V_r)(K_{mr}/K_{mf})(K_{m'r}/K_{m'f})$ (Dixon and Webb, 1964). The ethanol-ethanal pair has a K_{app} of 0.003, the hexan-1-ol-hexanal pair has a K_{app} of 0.012, and the 2-hexen-1-ol-2 hexenal has a K_{app} of 0.34. The constants favor ethanol and hexan-1-ol predominance but the equilibrium concentration of 2-hexen-1-ol and 2-hexenal approaches unity.

Activity with Mixed Substrate. Activities of citrus alcohol:NAD oxidoreductase with both ethanol and hexanol are shown in Table IV. With hexanol at 1/4 molar concentration of ethanol, activity was equal to the rate with ethanol alone. However, at equal molar concentration of hexanol and ethanol, activity was depressed to the rate with hexanol alone.

DISCUSSION

The alcohol:NAD oxidoreductase from orange juice vesicles is similar to the enzyme from pea seeds (Eriksson, 1968) in the

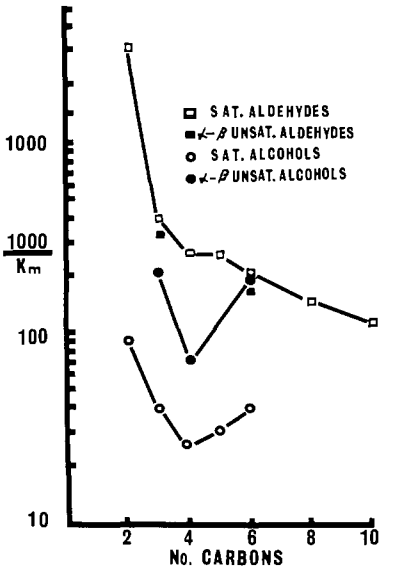

Figure 1. Substrate affinity of citrus alcohol:NAD oxidoreductase

Table I. Relative Maximum Velocity (V_f) of the Forward Reaction with Citrus and Yeast Alcohol:NAD Oxidoreductases

Substrate Concentration		Relative NAD-Reduction Activity	
	μmol/ml	Citrus	Yeast
ethanol	57	100	100
propan-1-ol	86	72	21
2-propen-1-ol	86	100	...
butan-1-ol	72	84	7.8
2-methylpropan-1-ol	72	26	...
2-buten-1-ol	72	115	...
pentan-1-ol	60	49	0
3-methylbutan-1-ol	60	66	...
4-penten-1-ol	60	82	...
hexan-1-ol	54	42	...
2-hexen-1-ol	54	168	21
3-hexen-1-ol	54	66	...
heptan-1-ol	23	14	0
octan-1-ol	21	7	0
nonan-1-ol	17	0	...
decan-1-ol	17	0	...

Specific activity of citrus oxidoreductase with ethanol was 6.0 μmol NAD reduced per min per mg protein. Specific activity of yeast oxidoreductase with ethanol was 90 μmol NAD reduced per min per mg protein.

Table II. Relative Maximum Velocity (V_r) of the Reverse Reaction with Citrus and Yeast Alcohol:NAD Oxidoreductases

Substrate Concentration		Relative NADH-Oxidation Activity	
	μmol/ml	Citrus	Yeast
ethanal	74	100	100
propanal	47	80	30
2-propenal	47	100	...
butanal	37	80	10
pentanal	33	80	10
hexanal	28	80	0
2-hexenal	28	85	...
heptanal	25	15	0
octanal	21	33	...
nonanal	19	33	...
2-nonenal	19	25	...
decanal	18	6	...

Specific activity of citrus oxidoreductase with ethanal was 15 μmol NADH oxidized per min per mg protein. Specific activity of yeast oxidoreductase with ethanal was 250 μmol NADH oxidized per mg protein.

Table III. Michaelis Constants of Citrus Alcohol:NAD Oxidoreductase for Forward (K_{mf}) and Reverse (K_{mr}) Reaction Substrates

	K_{mf} μmol/ml		K_{mr} μmol/ml
NAD ($K_{m'f}$)	0.14	NADH ($K_{m'r}$)	0.04
ethanol	11	ethanal	0.3
propan-1-ol	25	propanal	2.5
2-propen-1-ol	4.2	2-propenal	2.9
butan-1-ol	38	butanal	3.8
2-buten-1-ol	14	pentanal	3.8
pentan-1-ol	30	hexanal	4.9
hexan-1-ol	25	2-hexenal	5.6
2-hexen-1-ol	5.1	octanal	6.5
		decanal	8.4

K_m values were determined by the method of Lineweaver and Burk (1934) using dialyzed preparation from 45–55% $(NH_4)_2SO_4$ fraction.

Table IV. Oxidoreductase Activity with Ethanol and Hexanol

Substrate		
Ethanol μmol/ml	Hexanol μmol/ml	NAD Reduction μmol/min/mg protein
0	14	0.9
0	27	1.4
0	54	2.0
54	0	3.8
54	14	3.8
54	27	3.2
54	54	2.5

All reactions were initiated by adding 0.01 ml of the dialyzed 55–65% $(NH_4)_2SO_4$ fraction to the 3.0 ml reaction mixture.

large number of alcohols and aldehydes that are active substrates for the enzymes. Because many of the active alcohols and aldehydes in Tables I and II are present in orange juice (Nursten and Williams, 1967), the oxidoreductase probably catalyzes equilibrium reactions between these and between other alcohols and aldehydes in the fruit. Solubility of octan-1-ol, nonan-1-ol, and decan-1-ol limited the concentration of these alcohols below that required for maximum reaction rate. The concentration of hexan-1-ol for half-maximum reaction rate (K_{mf}) is 25 μmol/ml so that the K_{mr} of octan-1-ol, nonan-1-ol, and decan-1-ol would probably be higher. Only 21 μmol of octan-1-ol and 17 μmol of nonan-1-ol and decan-1-ol were used in the assay.

The α–β unsaturated alcohol, 2-hexen-1-ol, was the most reactive alcohol in the assay and the only one that had a higher relative activity than the aldehyde partner. It was also the only one with higher affinity than the aldehyde partner for the enzyme. This high activity and affinity of the alcohol results in a K_{app} that is near unity for the reaction between 2-hexenal and 2-hexen-1-ol. Because the other aldehydes have much greater enzyme affinities and activities than their alcohols, the equilibrium constants for all the other alcohol–aldehyde pairs greatly favor the reverse reaction or alcohol formation. Only the ethanol–ethanal pair has been quantitated in orange juice; the ethanol concentration is several hundred times that of ethanal (Bruemmer and Roe, 1970b). The qualitative data of Wolford and Attaway (1967) support the idea that equilibrium favors alcohol formation in the saturated series. They found all the alcohols from ethanol to decan-1-ol in orange juice essence, but propanal, butanal, and pentanal were not detected in the aldehyde series.

The ethanol–ethanal pair is the primary substrate for citrus alcohol:NAD oxidoreductase because ethanal has the highest affinity in the system. The other alcohols and aldehydes compete with each other and with ethanol and ethanal for the enzyme. In the fruit, substrate concentration and affinity for the enzyme probably determine the reacting substrate. Hexan-1-ol, which has about one half the affinity of ethanol, did not compete with ethanol in the oxidoreductase system at 25% of the molar concentration of ethanol (Table IV). But hexan-1-ol replaces some of the ethanol activity in the system at equal molar concentration. Replacement of ethanol activity by hexan-1-ol indicates that only one enzyme is involved in the activities with ethanol and hexan-1-ol. Until the oxidoreductase activities with the various alcohols can be identified with distinct protein fractions, we assume that the activities are for one enzyme with broad substrate specificity.

The equilibrium concentration of the alcohol and aldehyde in the equation alcohol + NAD = aldehyde + NADH depends on the concentration ratio of the coenzyme in the expression: (aldehyde)/(alcohol) = K_{app} (NAD)/(NADH). Because both alcohols and aldehydes take part in other equilibrium reactions in the intact fruit, this expression does not completely describe their dependence. For example, the ethanal/ethanol ratio declines in proportion to the decline in NAD/NADH ratio in grapefruit during maturation (Bruemmer, 1969; Bruemmer and Roe, 1970b) and in oranges and grapefruit during controlled atmosphere storage (Davis and Bruemmer, 1970), but it declines much more rapidly than the NAD/NADH ratio in oranges and grapefruit during anaerobic respiration (Bruemmer and Roe, 1969). The concentrations of other alcohol–aldehyde pairs have not been determined in citrus juices under these conditions. Because hexan-1-ol-hexanal and octan-1-ol-octanal are flavor and aroma contributing substances in citrus juices, we plan to examine their equilibrium-concentration dependence on the NAD/NADH ratio. If these alcohols and aldehydes have a concentration ratio dependence on the citrus alcohol-NAD oxidoreductase system in the intact fruit, procedures might be developed to control their concentration ratio for flavor improvement and off-flavor prevention.

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