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Geometric specificity of alcohol dehydrogenases and its potential for separation of *trans* and *cis* isomers of unsaturated aldehydes

(enzymatic separation of geometric isomers/enzymes as catalysts in organic chemistry/specificity of enzymes/oxidoreductases/cinnamic compounds)

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ABSTRACT **The geometric specificity of three different alcohol dehydrogenases (alcohol:NAD$^+$ oxidoreductase, EC 1.1.1.1) (from yeast, from horse liver, and from *Leuconostoc mesenteroides*) in the reduction of *trans*- and *cis*-cinnamaldehydes has been investigated. All three enzymes display a remarkable *trans* specificity: they react with the *trans* isomer 7 to 647 times faster than with its *cis* counterpart. Experiments with the enzymatic reduction of 3-phenylpropionaldehyde, a saturated analog of cinnamaldehyde, have revealed that whereas *trans*-cinnamaldehyde possesses the "right" configuration for the active centers of the alcohol dehydrogenases, the *cis* isomer apparently does not fit the active centers well. All three alcohol dehydrogenases studied also exhibit a marked *trans* specificity in the reaction with α-methylcinnamaldehyde. The geometric specificity of alcohol dehydrogenases can be used for the production of otherwise hard to synthesize *cis* isomers of unsaturated aldehydes from their readily available *trans* counterparts: *trans*-cinnamaldehyde was irradiated with ultraviolet light (which converted it to a mixture of *trans* and *cis* isomers) then treated with NADH and yeast alcohol dehydrogenase (which selectively reduces only *trans* aldehyde into the alcohol), and finally the mixture of *cis*-cinnamaldehyde and *trans*-cinnamyl alcohol was separated easily by preparative column chromatography.**

The separation of *trans* and *cis* isomers of unsaturated compounds is a classical problem in organic chemistry (1). In addition to its fundamental interest, this problem is also of great practical significance. Most functional properties of organic molecules are determined by their molecular geometry, and therefore these properties for *trans* and *cis* isomers are bound to be different. This can be illustrated by the following examples. Only *cis* isomers of substituted cinnamic acids possess plant-growth regulatory activity (2). Only *trans* diethylstilbestrol displays estrogenic activity (3). Balsamo *et al.* (4) have demonstrated that whereas *trans* isomers of *N*-alkyl-α,β-dimethylcinnamamides exhibit central nervous system-depressant activity, the corresponding *cis* isomers display central nervous system-stimulant activity. Particularly striking examples of dramatic differences in biological activity of *trans* and *cis* isomers can be found in the area of sex pheromones. These pheromones typically are long-chain unsaturated aldehydes, alcohols, or esters. In many cases one geometric isomer of a given pheromone is a sex attractant for one insect species, whereas its counterpart attracts another species (5).

In order to take advantage of the difference in properties of the geometric isomers of unsaturated compounds, one should be able to readily prepare the *trans* and *cis* isomers separately. Unfortunately, most conventional chemical syntheses of a given unsaturated compound usually yield just one particular isomer, either *trans* or *cis* (6).

The isomer produced can, however, be converted into a mixture of *trans* and *cis* isomers by irradiation with ultraviolet light (7). In principle, these mixtures can be separated by using such methods as fractional crystallization, partial precipitation, or chromatography. Unfortunately, the aforementioned techniques are very laborious, time-consuming, and inefficient and therefore are not suitable for preparative separations.

It occurred to us that enzymes can be employed for the separation of *trans* and *cis* isomers of unsaturated compounds. The rationale for this idea is as follows. X-ray studies of the tertiary structure of enzymes have revealed that in most cases active centers represent narrow clefts (openings) penetrating from the surface into the interior of the protein molecule (8). Therefore it is rather unlikely that the geometric isomers will fit into such a cleft equally well and both in the correct way. If this assumption is valid, one can expect different reactivities of *trans* and *cis* isomers to a given enzyme. This, in turn, can be a basis for the enzymatic separation: because one of the isomers will be enzymatically converted to a *new chemical functionality*, it should be easy to separate it from the unreacted second isomer.

Clearly, the success of this approach depends on finding geometrically (*trans* or *cis*) specific enzymes capable of acting on a wide range of substrates (in particular, unsaturated). Alcohol dehydrogenases (alcohol:NAD$^+$ oxidoreductase, EC 1.1.1.1.) catalyze the reduction of a number of carbonyl compounds to the corresponding alcohols and the oxidation of numerous alcohols to the corresponding aldehydes and ketones (9). Although stereospecificity of alcohol dehydrogenases has been extensively investigated (see refs. 10–14 for review), the question of their geometric specificity has never been addressed. In this work we have examined from this standpoint alcohol dehydrogenases from three different sources—horse liver, yeast, and *Leuconostoc mesenteroides*. Using enzymatic reductions of cinnamaldehydes as model reactions, we have discovered a remarkable geometric specificity of alcohol dehydrogenases: *trans* isomers are reduced by the enzymes up to a 1,000 times faster than their *cis* counterparts.

MATERIALS AND METHODS

Alcohol dehydrogenases from yeast and horse liver were purchased from Sigma and had specific activities of 330 units/mg of protein and 1.5 units/mg of protein, respectively. Alcohol dehydrogenase from *L. mesenteroides* was obtained from Boehringer Mannheim and had a specific activity of 29 units/mg of protein. One unit oxidizes 1 μmol of ethanol per min.

trans-Cinnamaldehyde and α-methylcinnamaldehyde were purchased from Aldrich, 3-phenylpropionaldehyde from Pfaltz & Bauer (Stamford, CT), NADH from Sigma, and silica gel G

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from Merck. All other chemicals used in this work were of the highest purity commercially available.

Commercial cinnamaldehyde and α-methylcinnamaldehyde represent almost exclusively *trans* isomers (15). This has been independently proven by us chromatographically as illustrated below. Commercial cinnamaldehyde was analyzed by thin-layer chromatography on silica gel, using benzene as a solvent and 2,4-dinitrophenylhydrazine in 2 M HCl as a developer (16). A single spot with an R_f value of 0.24 was obtained.

Ultraviolet irradiation of cinnamaldehyde and α-methylcinnamaldehyde was carried out in a Rayonet photochemical reactor. An aqueous solution of an aldehyde was placed in a quartz cuvette and irradiated with four 3.9-W lamps emitting ultraviolet light with a maximum at 3,000 Å. Such irradiation is known to isomerize a fraction of the *trans* isomer to its *cis* counterpart (7, 17). In one experiment, a 10 mM *trans*-cinnamaldehyde solution in water was irradiated for 2 hr, then extracted with ether and analyzed by thin-layer chromatography as described above. This resulted in two separate spots with R_f values of 0.24 and 0.31, which were assigned to the *trans* and *cis* isomers, respectively. Heating of such a mixture of the geometric isomers at 100°C results in a gradual conversion [thermal stereoisomerization (18)] of the *cis*-cinnamaldehyde to the more stable *trans* isomer.

Alcohol dehydrogenase-catalyzed reduction of aldehydes with NADH was studied spectrophotometrically by following reduction of the absorbance at 340 nm (e.g., see ref. 19) (the aldehydes used in this work do not absorb light at this wavelength). A typical reaction mixture had a volume of 3.0 ml and contained 40 μM aldehyde, 220 μM NADH, and various amounts of alcohol dehydrogenase (depending on its source) in 0.1 M sodium phosphate buffer (pH 7.0). In the case of irradiated mixtures, the total concentration of the aldehyde was chosen in such a way that the concentration of the *cis* isomer was 40 μM.

RESULTS AND DISCUSSION

In this work we have studied the kinetics of alcohol dehydrogenase-catalyzed reduction with NADH of *trans* and *cis* isomers of two aromatic unsaturated aldehydes, cinnamaldehyde (C_6H_5—CH=CH—CHO) and α-methylcinnamaldehyde (C_6H_5—CH=C(CH_3)—CHO). We have used three different alcohol dehydrogenases—from yeast, from horse liver, and from *L. mesenteroides*.

Curve a in Fig. 1 represents the time course of the reaction of *trans*-cinnamaldehyde with NADH catalyzed by yeast alcohol dehydrogenase (no appreciable reaction takes place in the absence of the enzyme). This curve can be used for spectrophotometric "titration" of the aldehyde: the change in the absorbance at 340 nm divided by the extinction coefficient of NADH [$\varepsilon = 6.2\ mM^{-1}\cdot cm^{-1}$ (20)] yields the concentration of *trans*-cinnamaldehyde in solution.

Irradiation of *trans*-cinnamaldehyde with ultraviolet light brings about photostereoisomerization of the aldehyde (7, 17), as was confirmed by the thin-layer chromatography described in *Materials and Methods*. Curve b in Fig. 1 shows the reaction kinetics of such a mixture of *trans*- and *cis*-cinnamaldehydes with yeast alcohol dehydrogenase. One can see that in this case a fast drop in absorbance (analogous to that of curve a) is followed by a very slow decrease, and eventually the absorbance reaches the same level as curve a. This biphasic kinetics clearly indicates that *cis*-cinnamaldehyde reacts with yeast alcohol dehydrogenase much slower than does the *trans* isomer. Extrapolation of the slow component of curve b to the time zero (the broken line in Fig. 1) affords determination of the fraction of the *cis* isomer in the irradiated aldehyde, which turned out to be 40%.

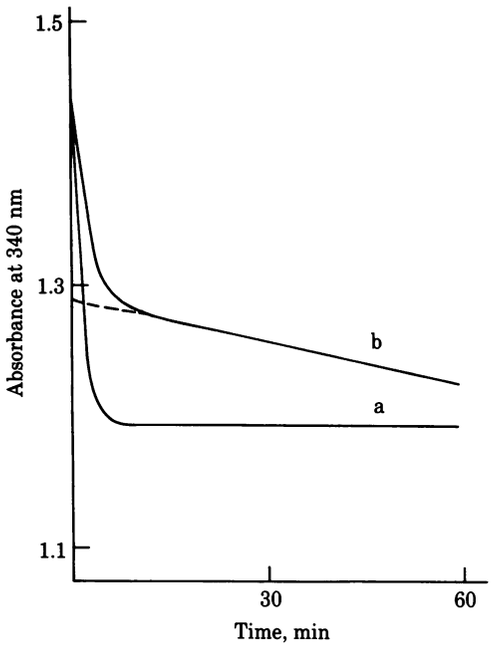

FIG. 1. Time course of yeast alcohol dehydrogenase-catalyzed reduction of *trans*-cinnamaldehyde (curve a) and photochemically produced mixture of *trans*- and *cis*-cinnamaldehydes (curve b), using NADH as an electron donor. Conditions: 40 μM cinnamaldehyde, 220 μM NADH, alcohol dehydrogenase at 33 units/ml, 0.1 M phosphate buffer (pH 7.0). In the case of curve b, 40 μM *trans*-cinnamaldehyde in 0.1 M phosphate buffer (pH 7.0) was irradiated by ultraviolet light for 30 min.

Fig. 1, curve b, also can be used for the measurement of the absolute rate of the enzymatic reduction of *cis*-cinnamaldehyde. The corresponding rate for the *trans* isomer can be measured in an experiment similar to that presented in curve a of Fig. 1 but at a much lower concentration of the enzyme. The rates obtained are presented in the first line of Table 1. One can see that *trans*-cinnamaldehyde is about 650 times more reactive to yeast alcohol dehydrogenase than is *cis*-cinnamaldehyde.

We have performed analogous experiments with horse liver and *L. mesenteroides* alcohol dehydrogenases. The corresponding data are shown in Table 1. Although both of these dehydrogenases exhibit a very significant geometric (*trans*) specificity, it is lower than for the yeast enzyme. Therefore, in order to determine the exact rates of the reduction of *cis*-cinnamaldehyde by the last two enzymes the following approach was

Table 1. Rates of reduction of *trans*- and *cis*-cinnamaldehyde and 3-phenylpropionaldehyde catalyzed by different alcohol dehydrogenases

Alcohol dehydrogenase	Reaction rate *v*, nmol/min per unit of enzyme: *trans*-Cinnamaldehyde	*cis*-Cinnamaldehyde	3-Phenylpropionaldehyde	*trans/cis*
Yeast	3.3	0.0051	5.7	647
Horse liver	7,590	1,160	14,400	7
L. mesenteroides	7.0	0.19	3.9	37

Conditions: 40 μM aldehyde (each isomer) and 220 μM NADH; the concentrations of the enzymes were varied to give conveniently measurable rates. For the experimental procedure in the reduction of *cis*-cinnamaldehyde catalyzed by the horse liver and *L. mesenteroides* alcohol dehydrogenases, see *Results and Discussion*.

employed: first, the mixture of the geometric isomers produced by ultraviolet irradiation was treated with the yeast alcohol dehydrogenase (which reduced all the *trans* isomer within a few minutes, see Fig. 1, curve a), and then either the horse liver or *L. mesenteroides* alcohol dehydrogenase was added. The initial rate of the enzymatic reduction of *cis*-cinnamaldehyde was measured and then the rate of the reduction due to the yeast enzyme (known from an independent experiment) was subtracted to give the sought-after rate for the horse liver or *L. mesenteroides* enzyme.

In an attempt to understand the mechanism of the observed *trans* specificity of alcohol dehydrogenases, we have measured the rates of the enzymatic reduction of the saturated analog of cinnamaldehyde, 3-phenylpropionaldehyde. In the latter (in contrast to cinnamaldehyde) there is a free rotation around the C_α—C_β bond. As one can see in Table 1, for all three alcohol dehydrogenases the rates of the enzymatic reduction of *trans*-cinnamaldehyde and 3-phenylpropionaldehyde are quite close (differing in either direction by less than 2-fold). This implies that the *trans* isomer of cinnamaldehyde happens to have the "right" configuration for the active centers. Inasmuch as the reactivity of *cis*-cinnamaldehyde to all three enzymes is much lower than that of both *trans*-cinnamaldehyde and 3-phenylpropionaldehyde, the *cis* isomer clearly has a "wrong" configuration for the active centers of the enzymes. Thus the geometric specificity of the alcohol dehydrogenases in this case is due to the fact that the *cis* isomer does not fit the active center well.

It was of obvious interest to examine how a variation in the aldehyde structure will affect the geometric specificity of alcohol dehydrogenases. To this end, we have studied the kinetics of the enzymatic reduction of *trans*- and *cis*-α-methylcinnamaldehydes. The results obtained are presented in Table 2. It can be seen that in the case of this aldehyde, all alcohol dehydrogenases tested also display a marked *trans* specificity.

The ability to enzymatically analyze the composition of the mixtures of geometric isomers (e.g., see Fig. 1, curve b) affords determination of spectral and other additive properties of the obscure (in this particular case the *cis*) isomer as illustrated below. The absorption spectrum of *trans* cinnamaldehyde in 0.1 M phosphate buffer (pH 7.0) was measured and then the solution was irradiated with ultraviolet light. The spectrum of the mixture of *trans* and *cis* isomers produced was measured, followed by determination of the *trans*-to-*cis* ratio in the mixture with yeast alcohol dehydrogenase. Finally, the spectrum of the *trans* isomer of the corresponding concentration was subtracted from the spectrum of the mixture, yielding the spectrum of *cis*-cinnamaldehyde. It was found that λ_{max} for *cis*-cinnamaldehyde is 280 nm and $\varepsilon_{280} = 10.0\ mM^{-1}\cdot cm^{-1}$ (as compared with $\lambda_{max} = 290$ nm and $\varepsilon_{290} = 26.7\ mM^{-1}\cdot cm^{-1}$ for the *trans* isomer). Similar experiments were conducted with α-methylcinnamaldehyde (employing horse liver alcohol dehydrogenase for the titration of the isomeric mixture) and the following parameters were found for the *cis* isomer: $\lambda_{max} = 280$ nm and $\varepsilon_{280} = 4.4\ mM^{-1}\cdot cm^{-1}$ (as compared with $\lambda_{max} = 284$ nm and $\varepsilon_{284} = 22.0\ mM^{-1}\cdot cm^{-1}$ for the *trans* isomer). One would expect such a reduction in λ_{max} and ε_{max} for the *cis* in comparison with the *trans* isomer due to a steric inhibition of resonance because of distortion from planarity, which increases the energy of the system.

Table 2. Geometric specificity of different alcohol dehydrogenases in the reduction of α-methylcinnamaldehyde

Alcohol dehydrogenase	Reaction rate v, nmol/min per unit of enzyme: *trans*-α-Methylcinnamaldehyde	Reaction rate v, nmol/min per unit of enzyme: *cis*-α-Methylcinnamaldehyde	v_{trans}/v_{cis}
Horse liver	2,240	1.82	1,230
Yeast	0.0293	0.00185*	16
L. mesenteroides	0.37	0.089*	4

Conditions: 40 μM aldehyde (each isomer) and 220 μM NADH; the concentrations of the enzymes were varied to give conveniently measurable rates.

* These rates were determined by using the same approach as described for cinnamaldehyde in *Results and Discussion* except using the horse liver (instead of yeast) enzyme for the initial reduction of the *trans* isomer.

An obvious application of the discovered geometric specificity of alcohol dehydrogenase is for preparative separation of *trans* and *cis* isomers of unsaturated aldehydes. For example, *trans*- and *cis*-cinnamaldehydes cannot be readily separated on a preparative scale because of the similarity in most of their physicochemical properties. However, treatment of the mixture with the yeast alcohol dehydrogenase for a short period of time should convert the *trans* isomer into a molecule with a new chemical functionality, namely cinnamyl alcohol. Therefore the enzymatic treatment transforms the problem of separation of *trans*- and *cis*-cinnamaldehyde into a much easier task, separation of *cis*-cinnamaldehyde from *trans*-cinnamyl alcohol.

We have experimentally proven the feasibility of such an approach. One liter of a 1 mM solution of *trans*-cinnamaldehyde in 0.1 M sodium phosphate buffer (pH 7.0) was UV irradiated for 2 hr (which resulted in conversion of 40% of the aldehyde into the *cis* isomer), followed by addition of 600 mg (0.8 mM) of NADH and 5,000 units of yeast alcohol dehydrogenase. The

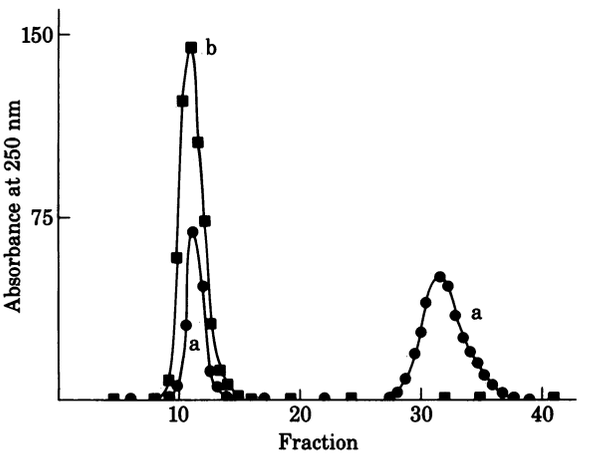

FIG. 2. Silica gel column chromatography of a 10 mM mixture of *trans*- and *cis*-cinnamaldehydes treated with the yeast alcohol dehydrogenase (curve a, ●) and a 10 mM untreated mixture of *trans*- and *cis*-cinnamaldehyde (curve b, ■). In the case of curve a, 1 liter of 1 mM *trans*-cinnamaldehyde in 0.1 M phosphate buffer (pH 7.0) was irradiated for 2 hr (to produce a mixture containing about 40% of the *cis* isomer), treated with NADH and the enzyme, and then extracted with 5 ml of chloroform. The chloroform extract was passed through the column. In the case of curve b, 10 ml of 10 mM *trans*-cinnamaldehyde in 0.1 M phosphate buffer (pH 7.0) was irradiated for 1.5 hr (to produce a mixture containing about 40% *cis* isomer) and extracted with 5 ml of chloroform. The chloroform extract was passed through the column. Conditions: 2.5 × 17 cm column packed with silica gel G and equilibrated with chloroform, flow rate 0.6 ml/min, fraction volume 5 ml. The second peak of curve a was positively identified as *trans*-cinnamyl alcohol by comparison of its spectral properties with those of an authentic sample obtained from Aldrich.

solution was stirred for 15 min, then extracted with 5 ml of chloroform and passed through a column packed with silica gel preequilibrated with chloroform. The resultant chromatogram is presented in Fig. 2 (curve a). One can see that an excellent resolution is obtained. For comparison, we chromatographed a mixture of *trans*- and *cis*-cinnamaldehyde in chloroform under the same conditions. As curve b in Fig. 2 shows, no resolution was achieved.

It should be pointed out that, by using recently developed NADH regeneration methods (see refs. 13, 21, and 22 for review and also refs. 23 and 24), the alcohol dehydrogenase-catalyzed separation of *trans* and *cis* isomers can in principle be carried out in the presence of only catalytic (as opposed to stoichiometric) amounts of the coenzyme.

In closing, it is worth noting that in addition to the remarkable *trans* specificity of alcohol dehydrogenases described in this paper, we have recently discovered and used for preparative separations a marked geometric specificity of two other enzymes, cow's milk xanthine oxidase (in the oxidation of β-arylacroleins to the corresponding carboxylic acids) (25) and pig liver carboxyl esterase (in the hydrolysis of methyl β-arylacrylates) (26). It appears that this may be a general phenomenon in enzymatic catalysis. If that is indeed the case, geometrically specific enzymes can be extremely useful for the production of particular geometric isomers of various pharmaceutical, agricultural, and fine chemicals.

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