# ADAMANTANONE AS A PROBE FOR THE DIMENSIONS AND CHARACTERISTICS OF THE SUBSTRATE BINDING POCKET OF CERTAIN ALCOHOL DEHYDROGENASES[1]

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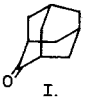
In this communication we illustrate how the rigid bicyclic molecule adamantanone (I)  may serve as a probe to delineate the dimensions of the substrate-binding pocket of several alcohol dehydrogenases and aid in differentiating between a fixed binding pocket (i.e. template type) and a conformationally mobile pocket whose precise shape depends upon a substrate induced "fit" (Koshland, 1962). The enzymes involved in this study are the 3α-hydroxysteroid dehydrogenase from Pseudomonas testosteroni (Marcus and Talalay, 1956), the 3β-17β-hydroxysteroid dehydrogenase from the same organism (Marcus and Talalay, 1956) and horse-liver alcohol dehydrogenase (ADH).

I.

The utility of adamantanone stems from the fact that all three enzymes accept and reduce simple (as well as complex) derivatives of cyclohexanone in two basic ring positions relative to a fixed coenzyme position. For example both the 3α- and the 3β-enzyme reduce steroids

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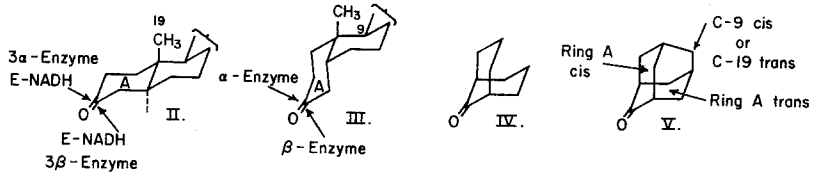
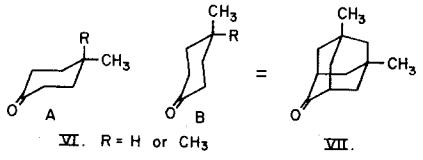
of either the A/B-trans (II) or A/B-cis (III) ring junction. Ring A of these two steroid types illustrates the two general positions for a derivative of cyclohexanone. By maintaining a fixed carbonyl




position and superimposing only ring A of the two steroids, part of the adamantanone molecule (V) is replicated. Addition of the carbon atom corresponding to C-19 of the trans-steroid or C-9 of the cis-steroid completes the adamantanone skeleton, as shown in V, since that carbon atom occupies the position of the methylene bridge of I. Therefore, when considered at a fixed position in space relative to a fixed coenzyme position, every carbon atom of the adamantanone skeleton is consistent with high enzymatic activity (i.e. satisfactory binding of the substrate) when incorporated into certain substrates.

The simpler molecules 4-methylcyclohexanone (VI, R = H) and 4,4-dimethylcyclohexanone (VI, R = $CH_3$) are substrates for both the 3α- and the 3β-enzyme (Ringold, et al., 1966) and for ADH (Merrit and Tomkins, 1959; Prelog, 1963, 1964; Graves, et al. 1965). These substrates form a substituted adamantanone (VII) when their rings, in the two basic positions VIA and VIB, are superimposed. The axial methyl group of the 4,4-dimethyl compound occupies the position of




VI. R = H or $CH_3$ VII.

the methylene bridge of I. Since 4-methylcyclohexanone has been shown (Graves et al., 1965) to undergo ADH catalyzed reduction in the two basic cyclohexanone ring positions, adamantanone again represents a skeleton in which every carbon atom is consistent with high enzymatic activity.

If the substrate-binding pocket is of the fixed template type, the following predictions may be made for the three enzymes. First, adamantanone should fit as a satisfactory substrate in each case. With the 3α- and 3β-enzymes, adamantanone should be a considerably better substrate than cyclohexanone. This follows because the addition to cyclohexanone of carbon atoms that correspond to the skeleton of the steroid nucleus increases substrate binding and hence the rate of reduction at non-saturating substrate concentrations. With ADH, in contrast to the steroid enzymes, substituents on the cyclohexanone ring have been shown to generally decrease the efficiency of substrate binding. From the rate inhibition factors that have been calculated in a previous publication (Graves et al., 1965), the rate of reduction of adamantanone with ADH catalysis should be slightly less than that of cyclohexanone.

Table I lists the relative reaction rates of adamantanone, cyclohexanone, 4-methylcyclohexanone and 4,4-dimethylcyclohexanone as determined by slope ratios from Lineweaver-Burk plots of $\frac{1}{c}$ vs $\frac{e}{v}$.[3]

With the 3α-enzyme adamantanone was reduced at 79x the rate of cyclohexanone. The effect of the axial methyl substituent of

[3] The slope is inversely proportional to the reaction rate and the comparison of ratios for two different substrates gives a direct measure of relative rates at identical low substrate concentration. All substrates in this study were run at concentrations much lower than $K_m$ values.

Table I

Relative Reduction Rates

| Substrate | Enzyme | | |
|---|---|---|---|
| | 3α | 3β | ADH |
| Cyclohexanone[1] | 1 | 1 | 1 |
| 4-Methylcyclohexanone | 4 | 3 | 0.4 |
| 4,4-Dimethylcyclohexanone | 7 | 27 | 0.5 |
| Adamantanone | 79 | 14 | 0.002 |

[1]Cyclohexanone is arbitrarily assigned a rate of unity.

the 4,4-dimethyl compound (which corresponds to the methylene bridge of adamantanone) can be shown to be a rate enhancement of 1.8x by comparing the 4-monomethyl and 4,4-dimethyl compound. Therefore the two basic rings combined into the single structure IV undergo reduction about 44x more efficiently than cyclohexanone. Thus the binding pocket of the 3α-enzyme is of sufficient size to simultaneously accommodate ring A of a cis- and of a trans-steroid; further most if not all of the carbon atoms of the fused ring system are simultaneously recognized and bound by the enzyme. This behavior strongly points to a template type substrate pocket for the α-enzyme although the possibility of a substrate-induced fit cannot be excluded with certainty.

With the 3β-enzyme, adamantanone was reduced only 14x faster than cyclohexanone and 0.5x the rate of 4,4-dimethylcyclohexanone. Since the axial methyl substituent of the latter contributes a rate factor of almost 10-fold, the calculated rate of the basic structure IV is only about 1.5x cyclohexanone. Therefore, although the binding pocket of the β-enzyme is of sufficient size to accommodate the structures I or IV, it is clear that the enzyme does not recognize

both rings simultaneously. Since these rings are individually recognized when contained in a cis- and in a trans-steroid, or in simpler molecules, it is strongly indicated that the binding pocket of the β-enzyme undergoes a substrate-induced fit and is not of the fixed template type.

The case of ADH appears quite clear-cut. Adamantanone was reduced at less than 1/500 the rate of cyclohexanone while the 4- and 4,4-dimethylcyclohexanones were reduced at about one-half the rate of the standard. Consequently, for all practical purposes the dimensions of the substrate recognition pocket are insufficient to accommodate adamantanone and presumably IV even though all carbon atoms of I are acceptable[4] to the enzyme when they are incorporated into other substrates. It can be concluded that the substrate receptor site of ADH is not of the fixed template type - instead, its shape must depend upon the particular substrate. Although stretching may be permitted within certain limits, the bulk of (I) has reached the point where binding can be accomplished only with severe distortion of the binding site. While Graves et al. (1965) have shown that it is possible to correlate substrate structure and the rate of enzymatic transformation with ADH, such calculations must be viewed with caution because of the possibility of substrate-

[4] Assuming that VIa and VIb are not significantly distorted from their normal chair conformation when they are bound by the enzyme. It should be noted that the carbonyl bond angle of adamantanone is slightly distorted compared to cyclohexanone (112.5° vs 116.3°) (Schleyer and Nicholas, 1961). While there is no way of evaluating the effect of the carbonyl bond angle on substrate binding it is unlikely that this 4° deviation is an important factor in the unreactivity of I.

induced fit. Similar considerations apply to the use of a diamond lattice for predicting acceptability of an unknown substrate. Although adamantanone is an unsatisfactory substrate it fits within the permitted portion of the lattice constructed by Prelog (1963, 1964) and by Rety (1963) for ADH substrates.

## Experimantal

3α- and 3β-Enzymes. Pseudomonas testosteroni ATCC 11996 (steroid adapted) was purchased from Worthington Biochemical Corp. as a dried powder and the 3α- and 3β-enzymes separated by the general procedures of Boyer, et al. (1965) and Squire, et al. (1964). The powder was sonically disrupted in 0.03 M phosphate buffer containing EDTA (0.001 M) and the cell-free preparation treated with protamine sulfate. Preliminary separation of the two enzymes was effected by ammonium sulfate precipitation and final separation by passage through Sephadex G-100. The central fractions of 3α-enzyme obtained in this manner were free of β-activity. The β-enzyme fractions contained some α-activity which was removed by pooling and concentration of the peak fractions followed by a second and in some cases, third separation on Sephadex. Such preparations were completely stable in solution for many weeks at cold-room temperature and were used as such for the kinetic runs described in this paper. With a variety of steroidal and non-steroidal substrates no difference in enzymatic behavior could be detected when enzyme at this stage of purity was compared to material further purified by column electrophoresis (Squire et al., 1964) or by chromatography on DEAE- or carboxymethyl-cellulose (Boyer, et al., 1965). The more highly purified enzyme was found to be generally unsatisfactory for extensive kinetic determinations because of its extreme instability in solution, a behavior previosly noted by Boyer et al.

Horse Liver Alcohol Dehydrogenase. Pietruszko, et al., (1966) have shown that crystalline ADH consists of five closely related enzyme forms, one of which contains significant steroid activity. The major zones (designated 3, 4 and 5 on the basis of electrophoretic mobility) have been separated (Pietruszko, et al., 1966) from the faster moving components by chromatography on carboxy-methyl cellulose and found to be free of steroid activity. This preparation was utilized for the present study. However, an enzyme preparation containing all five zones gave essentially identical results with the four substrates studied.

Kinetics. Reduction of the ketonic substrates was followed spectrophotometrically by the disappearance of the NADH maximum at 340 mμ. Relative reaction rates were determined by the slope ratios of Lineweaver-Burk plots of $\frac{1}{c}$ vs $\frac{e}{v}$. The assay system contained $NADH_2$ ($1.75 \times 10^{-4}$ M), phosphate buffer (0.03 M, pH 7.0) and the substrate contained in an organic solvent, total volume

3.0 ml. All runs were made with scale expansion on a Beckman DUR Spectrophotometer with a Gilford 2000 attachment. The concentration range of substrate and enzyme concentration are tabulated below. For the α- and β-enzyme the substrates were added in 0.1 ml dimethylformamide while for ADH the substrate was dissolved in 0.01 ml of methanol.

| | α-Enzyme | β-Enzyme | ADH |
|---|---|---|---|
| Cyclohexanone | 1.25 -5.0[1](710)[2] | 1.25 -5.0(170)[2] | 0.125 -1.0 (7.5)[3] |
| 4-Methyl- | 1.25 -5.0 (470) | 0.5 -2.0(170) | 0.125 -1.0 (7.5) |
| 4,4-Dimethyl | 0.5 -2.0 (470) | 0.5 -2.0(170) | 0.125 -1.0 (7.5) |
| Adamantanone | 0.125-0.5 (680) | 0.125-0.5(170) | 0.0625-0.25(75) |

[1]Substrate concentration in mg/3 ml.
[2]Enzyme concentration in units/3 ml. One enzyme unit is defined as an O.D. change of 0.001/min. in the standard assay described by Boyer et al. with androsterone and testosterone as α- and β-substrates respectively.
[3]Enzyme concentration in μg/3 ml.

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