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THE ENZYME "ALDEHYDE OXIDASE" IS AN IMINIUM OXIDASE. REACTION WITH NICOTINE $\Delta^{1'(5')}$IMINIUM ION

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SUMMARY: The second of the two reaction steps involved in the metabolic transformation of (-)-nicotine to (-)-cotinine (3) (i.e., the oxidation of the intermediate 2) is mediated mainly, if not solely, by the enzyme aldehyde oxidase (EC 1.2.3.1). Of the molecular species that constitute 2, nicotine $\Delta^{1'(5')}$ iminium ion (2a) appears to serve as the substrate. The enzyme has a strong affinity for 2a, as shown in a study on the inhibition of the oxidation of 3-(aminocarbonyl)-1-methylpyridinium chloride. This study gave a value of K_i = = 6 μM ; K_m = 2 μM (pH 7.4). Mainly in view of this finding, "iminium oxidase" seems to be a more adequate name than "aldehyde oxidase" for this enzyme.

The metabolic transformation of (-)-nicotine (1) into its principal metabolite (-)-cotinine (3) in mammals is a two-step reaction which proceeds via the intermediate 2 (1,2). The first reaction step seems to be hydroxylation catalyzed by the cytochrome P-450 system and the product formed initially should thus be 2b (1,3). An equilibrium mixture (2), the main components of which are the iminium ion 2a and the carbinolamine 2b, is then rapidly generated from 2b (3,4). Other species hypothetically present are 2c and 2d, but the amounts of these are so low that they could not be detected by NMR (4). The composition of 2 is dependent on pH and on other variables as well. Thus, at pH 7.4 the equilibrium level of 2a was found to be approximately 75 % in unbuffered aqueous solution but approximately 90 % in 0.2 M sodium phosphate buffer (4).

The second reaction step in the transformation of 1 into 3 was found by Hucker *et al.* to be mediated by a soluble enzyme which was suggested to be "an aldehyde oxidase" (1). In 1972, Hill *et al.* showed that 2, generated *in situ* from 1, mouse liver microsomes, 0_2, and NADPH, could indeed be oxidized to 3 by purified rabbit liver aldehyde oxidase (EC 1.2.3.1) (5).

A recently developed method for the synthesis (4) of a crystalline hydrodiperchlorate (4) of the iminium ion 2a has made it possible to investigate the enzymatic oxidation of 2 to 3 more closely. The results are reported below.

MATERIALS AND METHODS

The 2a hydrodiperchlorate (4) was synthesized as described previously (4). A control experiment demonstrated that autooxidation of 4 to 3 proceeded slowly; a yield of 3 of less than 0.5 % was obtained after occasional bubbling of 0_2 through a solution of 4 in sodium phosphate buffer, pH 7.4, for 30 min at

NMNA = 3-(aminocarbonyl)-1-methylpyridinium chloride

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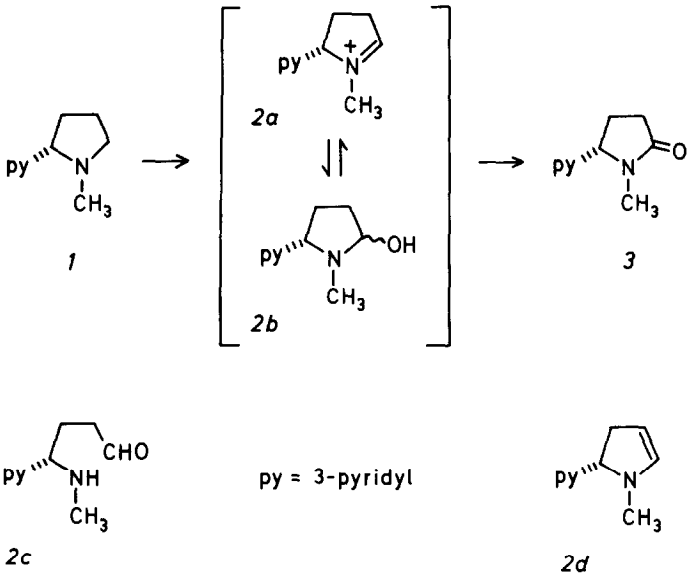

Fig. 1. Metabolic transformation of (-)-nicotine.

35 °C. For the enzymic reactions the hydrodiperchlorate of 2a and the corresponding dichloride served equally well as starting material. A fairly concentrated solution of 2a hydrodiperchlorate (4) (> 10 mM) decolorized 2,6-dichloroindophenol (i.e. 2,6-dichloro-4[(4'-hydroxyphenyl)imino]-2,5-cyclohexadien-1-one) within 20 min at 22 °C but, as seen spectropolarimetrically at 600 nm (6), no reduction of the indophenol by 4 took place during 10 min under the conditions used for the determination of K_m (Fig. 3). Reduction of potassium hexacyanoferrate(III) was faster; this compound was not suitable as an artificial electron acceptor. Due to the instability of aqueous solutions of 2a hydrodiperchlorate, these were kept on ice and used within 1 h. Determinations of the relative amounts of 2a and 2b at equilibrium in 0.2 M sodium phosphate buffer at pH 7.45, 8.45, and 9.20 were performed using the polarimetric method (4). The results are presented in Table 1. Milk xanthine oxidase (EC 1.2.3.2) was purchased from Sigma and assayed as previously described (7). The activity towards 2a hydrodiperchlorate was measured as for aldehyde oxidase (see below).

Purification of aldehyde oxidase. Rabbit liver aldehyde oxidase was partially purified (90 times with a 30 % yield) by carrying out four of the six purification steps described by Felsted et al.(8), with the exception (9) that the enzyme was eluted from the calcium phosphate gel with 0.05 M instead of 0.01 M phosphate buffer, pH 7.8. Protein determinations were performed according to Kalckar (10). The enzyme was assayed using 3-(aminocarbonyl)-1-methylpyridinium chloride (N^1-methylnicotinamide, NMNA), which is oxidized to a mixture of pyridones (8), and by following the increase in optical density at 300 nm as previously described (11). Changing the buffer from 0.05 to 0.2 M sodium phosphate did not affect the assay result. This assay procedure could not be applied, however, to the crude liver preparation (6,000 x g supernatant); so in this case the hexacyanoferrate(III) method (11) was used instead. To check this latter procedure it was also used together with the former in assays after the first purification step. Activity with 2a hydrodiperchlorate as substrate was determined by incubating a 0.42 mM solution with the enzyme preparations in 0.2 M sodium phosphate buffer, pH 7.4, for 4 min at 20-22 °C. Under these conditions the reaction was linear with time for at least 6 min. After 4 min, 0.5 ml 5 M NaOH and 0.62 µmol lidocaine were added and then NaCl(s) to saturation. The solution was extracted

with methylene chloride (4 x 2 ml) and the combined extracts dried with Na_2SO_4. The yield of cotinine 3 was determined by GLC (3 % JXR on Gas Chrom Q, 100-120 mesh, 0.2 x 180 cm) using the lidocaine as an internal standard. In all experiments described below the partially purified enzyme was used and, unless otherwise stated, the experiments were run at 20-22 °C in 0.2 M sodium phosphate buffer.

RESULTS AND DISCUSSION

It has been demonstrated by Hill et al. that aldehyde oxidase is capable of catalyzing the oxidation of 2 to 3 (5). However, it is also of interest to determine whether any other soluble enzyme is involved. To this end we measured the specific activities towards 3-(aminocarbonyl)-1-methylpyridinium chloride (NMNA) as well as towards 2a hydrodiperchlorate, during the partial purification of aldehyde oxidase. Since the ratio between the specific activities remained practically constant (1.0, 1.0, 1.4, 1.2, and 1.2 in the starting preparation and steps 1-4, respectively) during the purification procedure, it can be concluded that aldehyde oxidase is the main, if not the sole, enzyme mediating the oxidation of 2. Inhibitors of aldehyde oxidase, e.g., estradiol and p-benzoquinone (12), had a marked affect on the rate of oxidation of 2a hydrodiperchlorate (4) to 3. An 85 % inhibition was obtained with 0.06 mM estradiol and 0.42 mM 4. The effectiveness of p-benzoquinone as an inhibitor was comparable. Milk xanthine oxidase (EC 1.2.3.2) which, like aldehyde oxidase, is an unspecific molybdenum-containing enzyme (13), was found to be much less effective in oxidizing 2a hydrodiperchlorate (4) to 3. An amount of this enzyme which oxidized 2.4 μmol/min of xanthine oxidized approximately 1 nmol/min of 4, an amount not clearly distinguishable from that obtained in an autooxidation experiment. In striking contrast, a solution of aldehyde oxidase which oxidized 0.14 μmol/min of NMNA converted 0.17 μmol/min of 4 into 3.

Another question is which of the forms 2a - 2d is actually serving as substrate for the enzyme. Hill et al. (5) favored 2c but this hypothesis can hardly be correct, since it would be expected to lead to the formation of 4-methylamino-4-(3'-pyridyl)-butanoic acid, a compound which is not converted (14) into 3 under the conditions used. Instead, we have obtained evidence that 2a is the substrate. The evidence for this comes from a study on the pH dependence of the inhibitory action of 2a hydrodiperchlorate (4) on the oxidation of NMNA catalyzed by aldehyde oxidase (Fig. 2). It was initially observed that with 1.0 mM NMNA, 0.83 mM 4, and aldehyde oxidase the oxidation of NMNA was inhibited virtually completely, whereas the rate of cotinine formation was the same as when NMNA was omitted. In the investigation of the pH dependence, K_m values for NMNA were determined at pH 7.45, 8.45, and 9.20 (Table 1) and the inhibition exerted by a constant amount of 2a hydrodiperchlorate (4) on the oxidation of NMNA was studied at various concentrations of the latter and

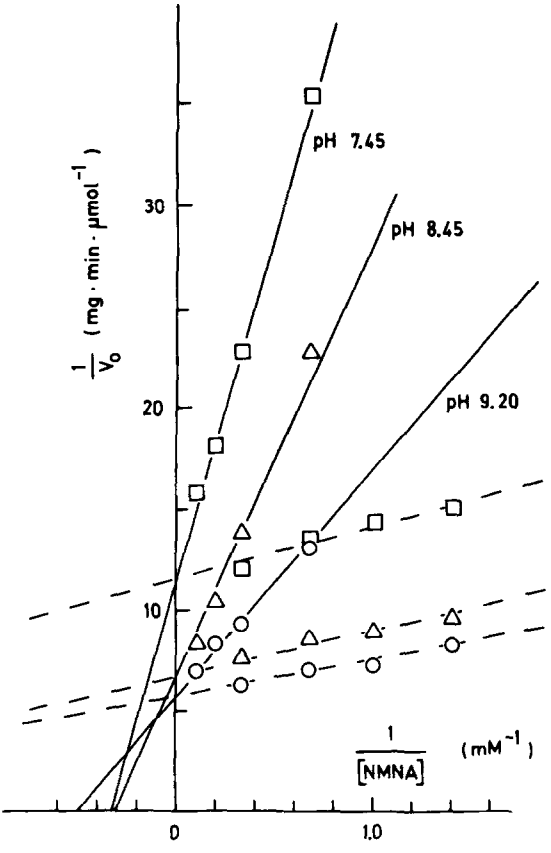

Fig. 2. Inhibition by 2a hydrodiperchlorate (4) (0.083 mM) of the oxidation of NMNA catalyzed by aldehyde oxidase at three different pH's. The measured results were analyzed using a program (BMDP3R, University of California, Los Angeles, USA) for unweighted non-linear regression analysis. The model fitted was that of a linear competitive inhibition. Constants K_m for NMNA and K_i for 4 are presented in Table 1.

at the same three pH values. The inhibition was found to be competitive and constants K_i were calculated for 4 from the kinetic data (Fig. 2). The relative amounts of 2a and 2b at equilibrium in 0.2 M phosphate buffer of various pH's have been estimated by measuring the optical rotation as a function of

Table 1. Calculation of sets of K_i for 2a and 2b. The sets are obtained thus: 6.7 x 0.85 = 5.7; 6.7 x 0.15 = 1.0, etc. The approximate constancy of the former set only is evidence that 2a rather than 2b is the true inhibitor. 4 = 2a hydrodiperchlorate.

pH	K_m for NMNA (mM)	K_i for 4 (µM)	Molar ratio 2a/2b	Calculated K_i for 2a (µM)	Calculated K_i for 2b (µM)
7.45	0.23 ± 0.05	6.7 ± 1.0	85/15	5.7	1.0
8.45	0.34 ± 0.08	10.0 ± 2.0	35/65	3.5	6.5
9.20	0.32 ± 0.03	16.0 ± 2.0	15/85	2.4	13.6

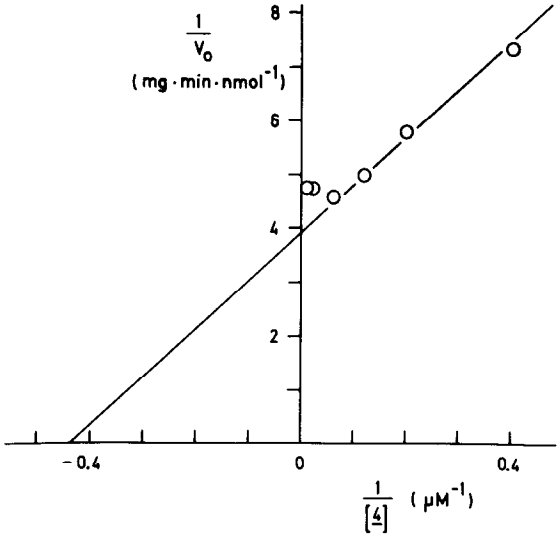

Fig. 3. Lineweaver-Burk plot of the oxidation of 2a hydrodiperchlorate (4) to 3 catalyzed by aldehyde oxidase at pH 7.4 (22 °C). The reaction rate was measured by following (at 600 nm) the aldehyde oxidase catalyzed reduction of 2,6-dichloroindophenol (6). A K_m of 2 μM was calculated from the intercept on the abscissa.

pH (Table 1)(4). Values thus obtained can be used to calculate two new sets of K_i for 2a and 2b (Table 1). Since apparent K_m and V_{max} values for NMNA only undergo a 50 % and a two-fold increase, respectively, in the pH interval studied, it is likely that the affinity of aldehyde oxidase for NMNA does not undergo any drastic change. If it is assumed that the same is true for the inhibitory form, then the set of K_i values calculated for 2a (Table 1) must be regarded as more likely than the set for 2b. Thus, 2a should be the inhibitory form and therefore, also the substrate for the enzyme.

The most striking feature of 2a in its interaction with aldehyde oxidase is its low K_m value of 2 μM (pH 7.4), obtained by measuring the dichloroindophenol reductase activity of the reduced form of the enzyme (Fig. 3). This is actually the lowest K_m recorded for any substrate for aldehyde oxidase; the lowest values previously reported are about one order of magnitude higher (9, 15). A K_m of 0.22 mM has previously been obtained for NMNA in 0.05 M phosphate buffer, pH 7.8, using the dichloroindophenol method (16).

Cotinine (3) does not inhibit the oxidation of 2a hydrodiperchlorate (4) and only weakly inhibits the oxidation of NMNA. At 1.0 mM NMNA and 2.1 mM 3 a 20 % inhibition was observed. Substrate inhibition seems to begin at about 0.3 mM 2a hydrodiperchlorate (4). The pH profile for the oxidation of 4 to 3 shows an optical region approximately between pH 6.8 and 9.5.

Compound 4 (2a hydrodiperchlorate) seems to be the first iminium ion that has been studied with respect to its interaction with aldehyde oxidase. Several compounds which contain an oxidizable carbon-nitrogen double bond as part of an aromatic system have, however, been studied before (9,15). It is to be expected that iminium ions are the principal intermediates in the metabolism of several saturated N-heterocyclic xenobiotics to the corresponding lactams and that aldehyde oxidase plays an important role in the oxidation of these intermediates.

As already pointed out by other investigators (9,17-19) the name "aldehyde oxidase" is inadequate for various reasons, but has been kept because good alternatives have been lacking. In view of the high affinity of the enzyme for 2a and for several aromatic compounds containing a carbon-nitrogen double bond moiety, the name "iminium oxidase" should be more descriptive.

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