The Analyst
FULL PAPER

Aromatic aldehydes as fluorogenic indicators for human aldehyde dehydrogenases and oxidases: substrate and isozyme specificity†

Piotr Wroczyński*[a] and Jacek Wierzchowski[b]

[a] *Department of Physical Chemistry, Faculty of Pharmacy, Medical School, 1 Banach St., PL-02097 Warsaw, Poland. E-mail: wropio@farm.amwaw.edu.pl*
[b] *Department of Organic Chemistry, Institute of Chemistry, University of Podlasie, 54 Trzeciego Maja St., PL-08110 Siedlce, Poland.*

Received 27th August 1999, Accepted 10th January 2000

Substrate properties of a number of potentially fluorogenic aromatic aldehydes of naphthalenes, phenanthrenes and anthracenes and of some coumarin aldehydes towards various forms of the human and rat aldehyde oxidase and dehydrogenase were examined using absorption and emission spectroscopy. It was demonstrated that recombinant human class 1 aldehyde dehydrogenase (ALDH-1) readily oxidizes naphthalene (except for those *ortho*-substituted), phenanthrene and coumarin aldehydes, whereas the class 3 enzyme (ALDH-3) from human saliva is active only towards 2-naphthaldehyde derivatives. The observed reaction rates in both cases are comparable to those of the best known substrates, and the K_m values are typically in the sub-micromolar range. Aldehyde oxidases (AlOx), which are present in mammalian liver, reveal much broader substrate specificity, oxidizing nearly all the compounds examined, including those of the anthracene series, with maximum activity in the micromolar range of substrate concentration. In rat liver, nearly all AlOx activity was located in the cytosolic fraction.

There are two main routes for aldehyde detoxication in mammals, namely the oxidation to carboxylic acids either by aldehyde dehydrogenases (ALDH, EC 1.2.1.3) or oxidases (AlOx, EC 1.2.3.1) and thiol-adduct formation, and various aldehyde species may undergo different transformations.[1] In humans, both ALDH and AlOx exist as mixtures of isozymes,[2,3] exhibiting differential specificity towards various aldehydes, and the activity of some of these isozymes has been shown to be critical for the therapeutic effectiveness of drugs. One of the best known examples of such a dependence is cancer chemotherapy utilizing the alkylating agent cyclophosphamide,[4] the effectiveness of which is dependent on ALDH activity in cancer cells. There is also growing evidence for selective expression of various ALDH and/or AlOx forms in both pathological and normal cells.[4] Hence the possibility of the selective measurement of the activities of individual ALDH and AlOx isozymes may be important for both diagnostic and therapeutic applications.

One possible approach to detecting and measuring the ALDH/AlOx activity is the application of potentially fluorogenic aromatic aldehydes. We have previously demonstrated that two main forms of the human cytosolic ALDH, namely ALDH-1 and ALDH-3, exhibit selective substrate properties towards two naphthaldehyde derivatives, *i.e.*, 7-methoxy-1-naphthaldehyde and 6-methoxy-2-naphthaldehyde, allowing the isozyme-specific measurement of their respective activities in tissue homogenates.[5] A similar approach is possible for various forms of AlOx (cytosolic or microsomal), and in fact, the activity of the latter is frequently observed in human organ homogenates, particularly in the liver.[3,4] In this investigation, we studied the substrate properties of other aromatic and selected heteroaromatic aldehydes towards various ALDH and AlOx forms, with the objective of identifying more fluorogenic substrates for these enzyme classes with potentially better spectral properties and improved selectivity for the individual isozymes.

† Presented as a poster at the VIIIth International Symposium on Luminescence Spectrometry in Biomedical Analysis, Las Palmas de Gran Canaria, Spain, May 1998.

Experimental

Enzymes, clinical and biological material

Purified recombinant human ALDH-1 was obtained by courtesy of Dr. R. L. Blakley (St. Jude's Hospital, Memphis, TN, USA). ALDH-3 isozyme activity was selectively measured using preparations of human saliva, where it is the only ALDH isozyme present.[6] Saliva samples were collected directly in test-tubes and diluted, ~3:1, with a cooled 50 mM pyrophosphate buffer (pH 8.1) containing 0.5 mM EDTA and 2 mM dithiothreitol (DTT). The precipitate that appeared was removed by centrifugation. The protein content in the supernatant was determined by the Bradford method,[7] and typical values were 0.2–1 mg mL^{-1}. The ALDH activity in such a preparation is stable for several days.[5]

Rat ALDH and AlOx activities were examined using crude rat liver homogenates. The rat liver fragments were stored at −80 °C. After thawing, the samples were homogenized in sucrose solution (0.25 M), buffered with 5 mM phosphate (pH 7.5) containing EDTA (1 mM) and DTT (2 mM). The homogenates were spun at 9000*g* to remove the mitochondrial fraction(s). The remaining supernatant (fraction S-9) contained, typically, 16–25 mg mL^{-1} of protein, as determined by the Bradford method.[7] This fraction was further purified by centrifugation at 50 000 rpm, and the resultant cytosolic fraction was stored at 4 °C. ALDH and AlOx activities were measured on the day of preparation.

Chemicals

The syntheses of 7-methoxy-1-naphthaldehyde (MONAL-71), 6-methoxy-2-naphthaldehyde (MONAL-62) and 6-dimethyla-

DOI: 10.1039/a906962c

mino-2-naphthaldehyde (DANAL-62) and the corresponding carboxylic acids have been reported elsewhere.[8,9] 5-Bromo-1-naphthaldehyde was obtained by the direct bromination of 1-naphthaldehyde[10] and 7-methoxycoumarin-4-caboxaldehyde by oxidation of 4-methyl-7-methoxycoumarin (Aldrich, Milwaukee, WI, USA) with selenium dioxide.[11] Aromatic and heteroaromatic carboxylic acids were obtained from the corresponding alcohols by oxidation with $KMnO_4$ in acetic acid.[12]

All the remaining enzymatic substrates were commercially available (Aldrich), and were used without purification. NAD^+, NADH and the protein microassay kit were obtained from Sigma (St. Louis, MO, USA). All other chemicals were of analytical-reagent grade. De-ionized, filtered water (Milli-Q system, Millipore Austria, Vienna, Austria) was used throughout. Aldehyde stock standard solutions (3 mM) were prepared in 30% aqueous acetonitrile and were stable for several weeks.[5,8]

Fluorimetric procedures

Typical fluorimetric assays[5,8] were run in 50 mM pyrophosphate–HCl buffer (pH 8.1) at 25 °C in the presence of 0.5 mM DTT and 0.5 mM EDTA. The concentration of ALDH-1 was adjusted to a value giving reaction rates near 50 nM min^{-1}. Crude liver homogenates were diluted 15–50-fold and saliva samples 10-fold. Typical substrate concentrations were 1–5 μM, which in most cases was sufficient to saturate the enzymes (see Results and discussion). The fluorescence of the carboxylate produced was observed at the maximum, with the excitation wavelength set above 300 nm (unless indicated otherwise), to minimize the protein fluorescence background. In clinical and biological samples, oxidase activity was measured for *ca.* 5 min prior to the addition of the coenzyme. Coenzyme NAD^+ was then added up to a concentration of 100 μM, which is sufficient to saturate both ALDH-1 and ALDH-3 human cytosolic isozymes.[5] After a few minutes of reaction, purified reaction product(s) were added to concentrations of 2–3 μM, providing internal standards for calculation of the absolute reaction rates.[8] If the reaction rate exceeded 100 nM min^{-1}, the oxidation was carried out until exhaustion of the substrate, and the final fluorescence provided the concentration standard. The reaction rate, v, was then calculated as follows:[8]

$$v = \frac{C_{st}}{F_{st}} \cdot \frac{\Delta F}{\Delta t}$$

where C_{st} is concentration of the fluorescence standard in μM, F_{st} the standard fluorescence and $\Delta F/\Delta t$ the fluorescence change during the reaction time (in minutes).

Enzymatic activity was expressed in international units per gram of protein, and calculated as v/[P], where [P] is protein concentration in the sample in grams per liter, and v is expressed in μM min^{-1}. Dehydrogenase activity was calculated as the difference between total activity and oxidase activity (*i.e.*, activity in the absence of NAD^+). The international unit is the amount of enzyme that catalyzes the synthesis of 1 μmol of the reaction product (carboxylate) per minute under saturating conditions.

For non-fluorescent or weakly fluorescent carboxylate products, the NAD^+-dependent oxidation of aldehydes was followed using the increase in NADH fluorescence. The fluorescence was excited at 360 nm and monitored at 460 nm, and NADH was added after 5–10 min of reaction up to a final concentration of 10 μM to provide an internal standard. The reaction rate was then calculated as above. Initial substrate concentrations were in this case 5–15 μM. Since the total absorbance change during all the assays (including the internal standard addition step) did not exceed 0.05, corrections for the inner filter effect were unnecessary.

The assays and spectral measurements were carried out on a Shimadzu (Kyoto, Japan) 5001 PC instrument. Quantum yields were determined relative to tryptophan (0.15) or quinine sulfate (0.55).

Other procedures

Spectrophotometric assays were performed to determine independently the basic kinetic parameters of various forms of ALDH. Initial substrate concentrations were determined spectrophotometrically. Reactions were monitored at wavelengths providing the largest change in molar absorptivity and the reaction rates were calculated as

$$v = \frac{1}{d\Delta\varepsilon} \cdot \frac{dA}{dt}$$

where dA/dt is absorbance change with time, d is the pathlength (1 cm) and $\Delta\varepsilon$ is the total change in molar absorptivity between substrates (aldehyde + NAD^+) and products (carboxylate + NADH), in L mol^{-1} cm^{-1}. Rates were next recalculated into μM min^{-1}. Since ALDH is an irreversible enzyme,[4] $\Delta\varepsilon$ can be calculated from the total absorbance change during the reaction, when NAD^+ is in excess. On several occasions, reaction rates were measured both spectrophotometrically and fluorimetrically,[5] providing results that were identical within the limits of error (3–5%).

Kinetic parameters for the enzymatic reactions were estimated using either progress curve analysis or, in a few most interesting cases, Lineweaver–Burke plots. In most cases, the progress curve analysis provided only upper limits for K_m estimations for the aldehyde substrates (see Tables 1 and 2). Since oxidation of the aldehydes by ALDH is irreversible, the respective K_m values can be deduced directly from the progress curves. By contrast, the reaction catalyzed by AlOx reveals non-classical kinetics (see Results and discussion and Table 3).

Results and discussion

Fluorescence properties of aromatic aldehydes and corresponding carboxylates

Fluorimetric determination of the ALDH activity typically involves measurements of the NADH produced. However, if the carboxylate reaction product is sufficiently fluorescent, it can also be utilized as an enzyme indicator, usually with much better sensitivity and some degree of selectivity towards various ALDH isozymes.[5] Aromatic and heteroaromatic aldehydes are good candidates for such indicators, if their substrate properties towards ALDH are sufficient. A similar strategy can also be applied to AlOx.

With few exceptions, aromatic aldehydes are known to be either weakly fluorescent or non-fluorescent in aqueous media,[13] whereas the corresponding carboxylates are in many cases strongly fluorescent.[5,8] Spectral properties of the aromatic aldehydes of naphthalenes, phenanthrenes and anthracenes and their oxidation products, investigated in this work, are summarized in Table 4. The fluorogenic character of 2-naphthaldehyde is illustrated in Fig. 1. This naphthaldehyde exhibits moderate fluorescence over the visible range, which after enzymatic oxidation is replaced by strong fluorescence of the carboxylate, centered at 355 nm. Similarly, oxidation of 6-dimethylamino-2-naphthaldehyde led to a very strongly emitting product (Table 4), whereas the carboxylates of phenanthrenes and anthracenes exhibit moderate fluorescence in the visible or near-UV range (Table 4). The fluorescence quantum yields of the carboxylates examined in this work are essentially excitation independent, as

Table 1 Relative rates of the enzymatic oxidation of aromatic and coumarin aldehydes by recombinant human ALDH-1, determined in the presence of 100 μM NAD^+ as a co-substrate at 25 °C. Rates were determined by the spectrophotometric method (expressed in μM min^{-1}) and the K_m values were estimated using progress curve analysis, unless indicated otherwise. Rates are normalized to benzaldehyde (100 μM) oxidation determined under identical conditions and using the same enzyme preparations

Substrate	Monitoring wavelength/nm	Initial concentration/μM	Relative rate (benzaldehyde = 100)	K_m/μM
1-Naphthaldehyde	352	53	82	< 2
4-Methoxy-1-naphthaldehyde	334	28	61	< 1
4-Dimethylamino-1-naphthaldehyde	366	47	28	< 5
5-Bromo-1-naphthaldehyde	320	33	36	< 5
7-Methoxy-1-naphthaldehyde	—	—	~ 80[a]	0.55[b,c]
2-Naphthaldehyde	330	48	70	⩽1[b]
6-Methoxy-2-naphthaldehyde	—	—	~ 90[a]	~ 0.02[a,b]
6-Dimethylamino-2-naphthaldehyde	—	—	~ 85[a]	~ 0.02[a,b]
7-Methoxycoumarin-4-carboxaldehyde	341	34	61	> 20
Phenanthrene-9-carboxaldehyde	316	27	50	< 2
Anthracene-9-carboxaldehyde	410	5	< 3	—

[a] Data from ref. 5; rates are relative to 100 μM acetaldehyde oxidation. [b] Estimation of K_m based on fluorimetric data. [c] Estimation of K_m based on Lineweaver–Burke analysis.[5]

Table 2 Relative rates (benzaldehyde = 100) and K_m values for the enzymatic oxidation of aromatic aldehydes by ALDH-3 from human saliva, as determined by fluorimetric assay at 25 °C. Assay conditions were 50 mM pyrophosphate buffer (pH 8.1), 0.5 mM EDTA, 0.5 mM DTT, with 100 μM NAD^+ as a co-substrate. Rates were determined in μM min^{-1} as described under Experimental, and normalized to the rate of benzaldehyde oxidation, determined spectrophotometrically under identical conditions and using the same enzyme preparations

Substrate	Initial concentration/μM	λ_{exc}/nm	Relative rate	K_m/μM
1-Naphthaldehyde	7	360[a]	< 27[a]	—
2-Naphthaldehyde	5	308[b]	54	0.46
6-Methoxy-2-naphthaldehyde	5	316	46[c]	0.24[c]
6-Dimethylamino-2-naphthaldehyde	4	320	21	1.7
7-Methoxy-1-naphthaldehyde	22	330	< 1	—
4-Methoxy-1-naphthaldehyde	5	360[a]	< 8[a]	—
4-Dimethyloamino-1-naphthaldehyde	1.5	360[a]	⩽9[a]	—
Phenanthrene-9-carboxaldehyde	12	302[b]	< 1	—
Anthracene-9-carboxaldehyde	5	360[a]	< 10[a]	—
Benzaldehyde	400	—	100	160[c]

[a] Rates determined by monitoring NADH fluorescence at 460 nm, with excitation at 360 nm. [b] Isosbestic points. [c] Data from ref. 5.

Table 3 Activities of aldehyde oxidase and dehydrogenase in rat liver homogenate fractions, examined with potentially fluorogenic aromatic and heteroaromatic aldehydes as substrates. Dehydrogenase activity was calculated as the difference between total oxidizing and oxidase activities (measured in the presence and absence of 100 μM NAD^+). Enzymatic activities are expressed in international units per gram of protein. Note that a substrate concentration of 5 μM gives nearly maximum oxidase activity for most of the aldehydes examined

		Substrate concentration/μM			
		Oxidase		Dehydrogenase	
Substrate	Liver homogenate fraction	1	5	1	5
2-Naphthaldehyde	Cytosolic	4.6	6.5	1.1	6.1
	S-9	nd[a]	6.6	nd	3.7
3-Methoxy-2-naphthaldehyde	Cytosolic	0.5	0.6	0.0	0.2
	S-9	0.7	0.8	0.1	0.3
1-Methoxy-2-naphthaldehyde	Cytosolic	0.4	0.4	0.0	0.0
	S-9	0.7	0.6	0.0	0.3
6-Methoxy-2-naphthaldehyde	Cytosolic	5.3	5.4	nd	nd
	S-9	7.1	~ 7	nd	16
7-Methoxy-1-naphthaldehyde	Cytosolic	3.3	3.2	0.0	0.2
	S-9	4.2	4.5	0.0	1.4
6-Dimethyloamino-2-naphthaldehyde	Cytosolic	2.2	3.4	nd	11.8
	S-9	2.0	3.5	nd	9.6
Anthracene-9-carboxaldehyde	Cytosolic	3.0	1.9	nd	0.0
	S-9	2.8	1.1	nd	0.0
Phenanthrene-9-carboxaldehyde	Cytosolic	0.8	0.5	4.0	3.8
	S-9	0.6	0.3	1.9	2.6

[a] nd = not determined.

revealed by the close similarity of the respective fluorescence excitation and absorption spectra (data not shown).

The fluorescence of aromatic aldehydes is known to be strongly solvent dependent,[13] virtually disappearing in non-hydroxylic media. This is of importance in assays of protein-rich samples, where non-polar aldehydes tend to associate with proteins, but in most cases this association did not affect the fluorescence background.[5,8] The only exception to this rule was 6-dimethylamino-2-naphthaldehyde, exhibiting strong, solvent-dependent fluorescence in organic solvents such as acetonitrile and dichloromethane (data not shown). This aldehyde, when added to protein-rich samples, caused a significant increase in the fluorescence background at the wavelengths critical for product detection (*ca.* 400–480 nm).

1-Naphthaldehyde, 4-methoxy-1-naphthaldehyde, 4-dimethylamino-1-naphthaldehyde and 5-bromo-1-naphthaldehyde emit weakly, and the corresponding carboxylates are of limited use as ALDH indicators because of insufficient fluorescence yields (Table 4), but were included in our investigation to characterize better the substrate requirements of the enzyme(s) studied. All the aromatic aldehydes exhibit UV absorption spectra markedly different from those of the corresponding carboxylates, allowing direct spectrophotometric assay of the enzyme activity.

7-Methoxycoumarin-4-carboxaldehyde exhibits strong fluorescence, which is diminished during enzymatic oxidation with ALDH-1 in the presence of NAD^+. The substrate fluorescence is strikingly similar to that of the corresponding alcohol,[14] and probably reflects hydration of this aldehyde in aqueous medium.

Substrate properties of the aromatic aldehydes towards various forms of ALDH

We examined the substrate specificity of two principal forms of the human cytosolic ALDH, ALDH-1 and ALDH-3 (known as a tumor-associated ALDH[4]) towards a number of potentially fluorogenic polyaromatic aldehydes of naphthalenes, phenanthrenes and anthracenes. Some of these compounds have been shown previously to be ALDH substrates,[8,15] but there are no systematic data on substrate/isozyme specificity. Our aim was to identify isozyme selective (or specific) fluorogenic substrates for individual ALDH and/or AlOx isozymes, or their classes, and to characterize the respective substrate specificities. The results, obtained by both spectrophotometric and fluorimetric methods, are summarized in Tables 1, 2 and 4.

The ubiquitous cytosolic ALDH-1 is known to oxidize many biogenic and exogenic aldehydes at essentially similar rates.[5,15,16] As evidenced in Table 1, it also oxidizes nearly all naphthaldehyde derivatives (except those *ortho*-substituted), and also those of the phenanthrene series, but not the anthracene series. By contrast, the oxidizing activity of ALDH-3 from human saliva is limited primarily to the 2-naphthaldehyde series (Table 2).

Kinetic parameters for the oxidation of substituted 1- and 2-naphthaldehydes and phenanthrene-9-carboxyaldehyde by recombinant human liver ALDH-1 were estimated using a progress curve analysis, with reaction rates determined by the spectrophotometric and, wherever possible, also fluorimetric assays. At the initial substrate concentration of *ca.* 30 μM, the NAD^+-dependent enzymatic oxidation reactions were of the zero-order type (straight lines) during the complete reaction course, *i.e.*, until substrate exhaustion (*cf.*, Fig. 1, inset). This indicates very low values of the Michaelis constants (K_m), in most cases < 1 μM (Table 1). Only with very low initial substrate concentrations (or the order of 0.1 μM) and using the fluorimetric method could some curvature in the progress curve be observed (data not shown), allowing estimation of the K_m values for the most fluorogenic 2-naphthaldehyde and 6-dimethylamino-2-naphthaldehyde (Table 1). Exceptionally, for

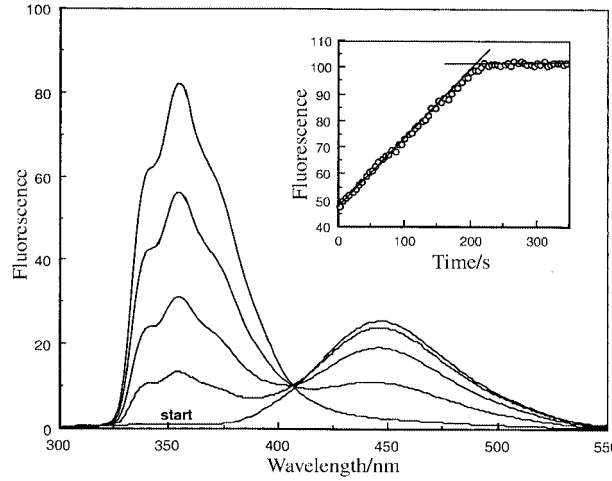

Fig. 1 Change of fluorescence spectrum during the enzymatic oxidation of 10 μM 2-naphthaldehyde by purified human aldehyde dehydrogenase (ALDH-1) in the presence of 100 μM NAD^+. The excitation wavelength was 280 nm and the reaction carried out in 50 mM pyrophosphate buffer (pH 8.1) at 25 °C. Inset: complete progress curve for the same reaction, monitored fluorimetrically at 360 nm, with initial substrate concentration 1.5 μM. Note the strictly zero-order character of the reaction kinetics virtually until exhaustion of the substrate.

Table 4 Summary of spectral properties of polyaromatic and heteroaromatic aldehydes examined in this work and their substrate properties towards various forms of aldehyde dehydrogenase and oxidase

Aldehyde substrates			Carboxylate fluorescence			Substrate properties towards		
Substrate	Solubility in water	Fluorescence in water, λ_{max}/nm	λ_{exc}/nm	λ_{max}/nm	Quantum yield	Human ALDH-1	Human ALDH-3	Rat AlOx (cytosolic)
1-Naphthaldehyde[a]	+	No			0	+[c]	—	+
4-Methoxy-1-naphthaldehyde[a]	+	Weak, 420		380	< 0.01	+[c]	—	+
4-Dimethylamino-1-naphthaldehyde[a]	+	No			0	+[c]	Weak	+
2-Naphthaldehyde[a]	+	Moderate, 450	280–310	355	0.19	+[c,d]	+	+
1-Methoxy-2-naphthaldehyde	±	No	325	360	0.06	—	—	±
3-Methoxy-2-naphthaldehyde	±	No	310	380	~ 0.2	±	?	±
6-Dimethylamino-2-naphthaldehyde[a]	±	Moderate, 530	320	440	0.82	+[c]	+	+
Phenanthrene-9-carboxyaldehyde[a]	±	Weak	290	370	0.09	+[c,d]	—	±
Anthracene-9-carboxyaldehyde[a]	—[b]	No	280	410	~ 0.05	—[d]	—	+
7-Methoxycoumarin-4-carboxaldehyde	—[b]	Strong	280	440	< 0.01	+[c,d]	—	+

[a] Commercially available. [b] Investigated in the presence of 2% acetonitrile. [c] Spectrophotometric assay. [d] Fluorimetric assay.

7-methoxycoumarin-4-carboxaldehyde, the estimated Michaelis constant was above 20 μM, as deduced from a nearly first-order reaction course observed with an initial substrate concentration of *ca.* 35 μM (not shown). The reaction rates are in most cases comparable to those of the best known ALDH-1 substrates, benzaldehyde and/or acetaldehyde (Tables 1 and 2), reflecting the low specificity of the enzyme.

Another cytosolic form of human ALDH, ALDH-3, located primarily in the stomach and saliva, and undergoing strong induction in certain tumors,[4] was previously described as specific for the long-chain aliphatic aldehydes.[17] In a previous paper[5] we showed that the fluorogenic 6-methoxy-2-naphthaldehyde is one of the best substrates for this isozyme. As is evident from Table 2, the commercially available, highly fluorogenic 2-naphthaldehyde and the most fluorogenic 6-dimethylamino-2-naphthaldehyde (DANAL-62) are also oxidized by ALDH-3 from human saliva with good rates. The apparent K_m values for the oxidation of these were calculated from the Lineweaver–Burke plot, using fluorimetric assay. The resultant values (K_m = 0.46 and 1.7 μM, respectively) are comparable to that obtained previously for 6-methoxy-2-naphthaldehyde (0.24 μM)[5] and much lower than those of aliphatic ALDH-3 substrates.[17] Both compounds are, consequently, very good indicators of both ALDH-1 and ALDH-3 activity. Thanks to the low K_m values, both substrates could be applied in low micromolar concentrations, so the inner filter effects can be neglected in rate calculations (see Experimental). An improved selectivity towards ALDH-3 can be achieved using $NADP^+$ instead of NAD^+ as a cofactor,[5] since ALDH-1 does not accept the former coenzyme, whereas ALDH-3 does.[4]

Possible interference with ALDH determinations by the present method may come from alcohol dehydrogenase (ADH, EC 1.1.1.1), since nearly all aromatic alcohols exhibit strong fluorescence.[18] Inhibitors of ADH such as pyrazole derivatives are typically employed in standard ALDH assays,[4] but we have shown in several instances[5,8] that 4-methylpyrazole does not affect ALDH measurements by the fluorimetric method by more than 10%.

Oxidation of aromatic aldehydes by aldehyde oxidase

Aldehyde oxidases (AlOx) are low-specificity enzymes that catalyze the oxidation of aldehydes, and many other compounds, in the presence of molecular oxygen.[3] In mammalian organisms, they are located mainly in cytosolic and microsomal fractions of some organs, primarily the liver. Their presence is manifested by slow oxidation of the aromatic substrates in the absence of NAD^+, and is regarded as the primary limitation of the sensitivity of ALDH determinations in biological samples by fluorimetric methods.[5] AlOx activity can occasionally be detected in human serum in pathological states (to be published elsewhere), although in human tissue it is generally lower than in rodents.[3] We therefore used rat liver homogenates (S-9 and cytosolic fractions) as a source of aldehyde oxidases.

Our preliminary observations showed that most of the aldehydes investigated are in fact oxidized by both S-9 and cytosolic fractions of rat liver in the absence of NAD^+, but these reactions do not obey simple Michaelian kinetics, showing evidence of substrate inhibition above concentrations of 5–10 μM, and sometimes even less (*cf.* Table 3). Therefore, we decided to examine the substrate properties of the aromatic aldehydes using a concentration of 5 μM, which is close to the maximum activity for most of the substrates. The results, simmarized in Table 3, show that both the cytosolic and microsomal AlOx from rat liver exhibit low specificity, oxidizing all the investigated aromatic aldehydes, including anthracene carboxaldehyde and *ortho*-substituted methoxynaphthaldehydes, which are resistant to ALDH. Most of the observed activity was clearly located in the cytosolic fraction (*i.e.*, specific activities of the cytosolic and S-9 fractions were similar).

The oxidation of anthracene-9-carboxyaldedyde by AlOx leads to a moderately fluorescent antharene-9-carboxylate (λ_{max} = 408 nm), which is not a substrate for ALDH, and therefore can be a good and selective indicator of the cytosolic and/or microsomal[18,19] AlOx activity (Table 4). By contrast, there is no strictly specific substrate for ALDH which would be resistant to AlOx, but the moderately fluorogenic phenanthrenealdehyde shows relatively good selectivity for the former enzyme (Table 4).

The data presented in Table 3 indicate that substrate concentrations of 5 μM, which are sufficient to saturate human ALDH, do not always saturate rat liver ALDH. This is due to the fact that rat liver contains very little, if any, of ALDH-1, and probably two forms of ALDH-3, distinct from human ALDH-3.[4] Therefore, assay of rat ALDH would require higher concentrations of the fluorogenic aldehydes.

Concluding remarks

The unbiquitous human ALDH-1 isozyme readily oxidizes many aromatic aldehydes, including those of the naphthaldehyde, phenanthrenealdehyde and coumarinaldehyde series. In most cases, the K_m values are in the sub-micromolar region, and the reaction rates are comparable to those of the best ALDH substrates (acetaldehyde, benzaldehyde). Two new highly fluorogenic substrates, 2-naphthaldehyde and 6-dimethylamino-2-naphthaldehyde, complement those described previously.[5,8] The previously unnoticed susceptibility of complex polyaromatic aldehydes (such as phenanthrenealdehyde) to oxidation by ALDH-1 may lead to the development of ALDH substrates suitable for fluorescence microscopy. In contrast to the fluorogenic ALDH substrates used by others,[18,20] the aldehydes described in this work are stable in aqueous acetonitrile solution.[5,8]

ALDH-3 from human saliva (which is similar to, if not identical with, the 'tumor-associated' ALDH[4]) exhibits considerable specificity toward aromatic aldehydes, effectively oxidizing those of the 2-naphthaldehyde series, but being inactive towards 1-naphthaldehydes and higher polyaromatic aldehydes. The activity of this isozyme can be measured selectively thanks to its unique ability to use both NAD^+ and $NADP^+$ as co-substrates.[5,17] The commercially available 2-naphthaldehyde and highly fluorogenic 6-dimethylamino-2-naphthaldehyde (DANAL-62) are proposed as new substrates for the fluorimetric assay of this isozyme.

It is known that another form of human ALDH, the mitochondrial ALDH-2, preferentially oxidizes aliphatic aldehydes,[4] but some aromatic aldehydes are also oxidized by this enzyme, albeit slowly.[15,21] We have previously found that fluorogenic substrates of the 1-naphthaldehyde series, such as 7-methoxy-1-naphthaldehyde (MONAL-71[5,8] are very poor substrates of the human ALDH-2, and are totally resistant to ALDH-3 (*cf.* Table 2), can therefore be regarded as selective substrates for ALDH-1. It should be noted, however, that several previously unknown forms of human ALDH have been isolated in the last few years.[2] so the specificity of all the ALDH substrates must be checked against these new isozymes.

Both cytosolic and microsomal aldehyde oxidases from rat liver exhibit low specificity, oxidizing all the investigated aromatic aldehydes, including anthracenecarboxaldehyde and *ortho*-substituted methoxynaphthaldehydes, which are resistant to ALDH oxidation, so that AlOx activity can be measured selectively in biological samples. We hope that fluorogenic aromatic aldehydes can be also used to distinguish various forms of human AlOx,[3] but this goal required thorough

examination of individual isozymes, which were not at our disposal.

Acknowledgements

This work was supported by the State Committee for Scientific Research (KBN), grant No. 6P04A 043 12, and by the Medical School, Warsaw, projects I-A/44 and II-A/68.

References

1 N. E. Sladek, C. L. Manthey, P. A. Maki, Z. Zhangand G. J. Landkammer, *Drug Metab. Rev.*, 1989, **20**, 697.
2 A. Yoshida, *Eur. J. Biochem.*,1998, **251**, 549.
3 C. Beedham, *Pharm. World Sci.*, 1997, **19**, 255.
4 R. Lindahl, 1992, *Crit. Rev. Biochem. Mol. Biol.*, 1992, **27**, 283.
5 J. Wierzchowski, P. Wroczyński, K. Laszuk and E. Interewicz, *Anal. Biochem.*, 1997, **245**, 69.
6 L. E. Dyck, *Alcohol. Clin. Exp. Res.*, 1995, **19**, 420.
7 M. M. Bradford, *Anal. Biochem.*, 1976, **72**, 248.
8 J. Wierzchowski, E. Interewicz, P. Wroczyński and I. Orlańska, *Anal. Chim. Acta*, 1996, **319**, 209.
9 J. Wierzchowski, W. P. Dafeldaecker, B. Holmquist and B. L. Vallee, *Anal. Biochem.*, 1989, **178**, 57.
10 P. Ruggli and R. Preuss, *Helv. Chim. Acta*, 1941, **24**, 1345.
11 S. D. Joshi and R. N. Usgaonkar, *Indian J. Chem.*, 1982, **21B**, 399.
12 W. Carruthers, *Some Modern Methods of Organic Synthesis*, Cambridge University Press, Cambridge, 3rd edn., 1986, ch. 4.
13 I. P. Crowell and C. J. Varsel, *Anal. Chem.*, 1963, **35**, 189.
14 J. Wierzchowski, *Lab. Diagnost. (Warsaw)*, 1991, **27**, 29.
15 A. A. Klyosov, L. Rashkovetsky, M. K. Tahir and W.-M. Keung, *Biochemistry*, 1996, **35**, 4445.
16 W. Ambroziak and R. Pietruszko, *J. Biol. Chem.*, 1991, **266**, 13011.
17 L. C. Hsu, W.-C. Chang, A. Shibuya and A. Yoshida, *J. Biol. Chem.*, 1992, **267**, 3030.
18 G. G. Gilbault, in *Practical Fluorescence*, ed. G. G. Gilbault, Marcel Dekker, New York, 1990, ch. 12.
19 K. Watanabe, M. Ozaki, T. Matsunaga, I. Yamamoto and H. Yoshimura, *Anal. Biochem.*, 1991, **198**, 134.
20 R. A. Deitrich, *Biochem. Pharmacol.*, 1966, **15**, 1911.
21 A. A. Klyosov, *Biochemistry*, 1996, **35**, 4457.

Paper a906962c