

# Monitoring of fatty aldehyde dehydrogenase by formation of pyrenedecanoic acid from pyrenedecanal

Markus A. Keller,\* Katrin Watschinger,\* Georg Golderer,\* Manuel Maglione,<sup>†</sup> Bettina Sarg,<sup>§</sup> Herbert H. Lindner,<sup>§</sup> Gabriele Werner-Felmayer,\* Alessandro Terrinoni,\*\* Ronald J. A. Wanders,<sup>††</sup> and Ernst R. Werner<sup>1,\*</sup>

Division of Biological Chemistry\* and Division of Clinical Biochemistry,<sup>§</sup> Biocenter, Innsbruck Medical University, A-6020 Innsbruck, Austria; Center of Operative Medicine,<sup>†</sup> Department of Visceral, Transplant and Thoracic Surgery, Innsbruck Medical University, A-6020 Innsbruck, Austria; Istituto Dermopatico dell'Immacolata-Istituto di Ricovero e Cura a Carattere Scientifico (IDI-IRCCS) Biochemistry Laboratory,\*\* Department of Experimental Medicine, University of Tor Vergata, I-00133 Rome, Italy; and Laboratory of Genetic Metabolic Diseases,<sup>††</sup> Academic Medical Center at the University of Amsterdam, 1105 AZ Amsterdam, The Netherlands

**Abstract** Fatty aldehyde dehydrogenase (EC 1.2.1.48) converts long-chain fatty aldehydes to the corresponding acids. Deficiency in this enzyme causes the Sjogren Larsson Syndrome, a rare inherited disorder characterized by ichthyosis, spasticity, and mental retardation. Using a fluorescent aldehyde, pyrenedecanal, and HPLC with fluorescence detection, we developed a novel method to monitor fatty aldehyde dehydrogenase activity by quantification of the product pyrenedecanoic acid together with the substrate pyrenedecanal and possible side products, such as aldehyde adducts. As shown with recombinant enzymes, pyrenedecanal showed a high preference for fatty aldehyde dehydrogenase compared with other aldehyde dehydrogenases. The method allowed detection of fatty aldehyde dehydrogenase activity in nanogram amounts of microsomal or tissue protein and microgram amounts of Sjogren Larsson syndrome patients' skin fibroblast protein. It could successfully be adapted for the analysis of fatty aldehyde dehydrogenase activity in gel slices derived from low-temperature SDS-PAGE, showing that fatty aldehyde dehydrogenase activity from solubilized rat liver microsomes migrates as a dimer. **■** Thus, monitoring of pyrenedecanoic acid formation from pyrenedecanal by HPLC with fluorescence detection provides a robust and sensitive method for determination of fatty aldehyde dehydrogenase activity.—Keller, M. A., K. Watschinger, G. Golderer, M. Maglione, B. Sarg, H. H. Lindner, G. Werner-Felmayer, A. Terrinoni, R. J. A. Wanders, and E. R. Werner. **Monitoring of fatty aldehyde dehydrogenase by formation of pyrenedecanoic acid from pyrenedecanal.** *J. Lipid Res.* 2010. 51: 1554–1559.

**Supplementary key words** Sjogren Larsson Syndrome • fatty aldehyde metabolism • low-temperature SDS gel • dimer • long-chain aldehyde dehydrogenase

The 55 kDa membrane-bound enzyme fatty aldehyde dehydrogenase (EC 1.2.1.48) converts long-chain fatty aldehydes to the corresponding acid in an NAD-dependent reaction. This oxidation is of great physiological importance since free aldehydes are toxic to cells because of their high reactivity with free amino groups of lipids and proteins (1). The gene coding for fatty aldehyde dehydrogenase, ALDH3A2, lies on human chromosome 17 and has been associated with a genetically inherited disorder, Sjogren Larsson Syndrome (SLS) (2). More than 70 mutations in the ALDH3A2 gene have been described that cause this autosomal recessive disease (3). SLS patients suffer from ichthyosis, spasticity, tetraplegia, and mental retardation (4). Biochemical diagnosis of SLS is achieved through measurement of residual fatty aldehyde dehydrogenase activity in patients' fibroblasts by an enzyme assay. An assay widely used is based on the quantification of the increase in NADH concentration caused by the reduction of the cofactor NAD by fatty aldehyde dehydrogenase along with the oxidation of an unlabeled aldehyde, added as substrate to the assay mixture (5).

Another assay for the quantification of fatty aldehyde dehydrogenase monitors phytenic acid formed from phytol and subsequent analysis of the product by gas chromatography-mass spectrometry (6). An alternative fatty aldehyde dehydrogenase quantification is based on the use of a radioactively labeled long-chain fatty alcohol (1).

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<sup>1</sup>To whom correspondence should be addressed.  
e-mail: ernst.r.werner@i-med.ac.at

Recently, an additional assay was published that measures the conversion of a long-chain fatty alcohol (22-hydroxydocosanol) into the corresponding long-chain fatty acid and quantifies the product 22-hydroxydocosanoic acid by electrospray ionization-mass spectrometry (ESI-MS) (7).

In this work, we present a reversed phase HPLC method using pyrenedecanal as fluorescently labeled substrate for fatty aldehyde dehydrogenase and quantification of the fluorescent pyrenedecanoic acid product formed. This novel assay allows quantification of products and by-products in samples of patients and requires 10–50 times less protein than previous assays.

## MATERIALS AND METHODS

### Preparation and solubilization of microsomal fatty aldehyde dehydrogenase from rat liver

All steps were performed at 4°C. Male Sprague Dawley rat livers (250 g) were washed and homogenized with an Omni mixer (Sorvall, Newtown, CT) in 375 ml preparation buffer (100 mM Tris HCl, pH 7.6, 0.25 M sucrose, and 1 mM PMSF). After centrifugation at 3,000 *g* for 10 min, the supernatant was centrifuged at 13,000 *g* for 20 min. Microsomes were pelleted at 40,000 *g* for 1 h, and pellets were washed with preparation buffer and then resuspended in 10 ml of storage buffer (100 mM potassium phosphate, pH 7.5, 1 mM EDTA, and 1 mM DTT), yielding a total protein concentration between 10 and 20 mg/ml. Microsomes were stored at –75°C until used. Microsomes were washed for 30 min in an equivalent volume of washing buffer [20 mM potassium phosphate, pH 6.5, 1.5 M NaCl, 10% (v/v) glycerol], centrifuged for 30 min and 200,000 *g*, and then solubilized in 20 mM potassium phosphate, pH 6.5, 1 M NaCl, 0.15% Triton X-100 (reduced form), 10% (v/v) glycerol, and 1 mM DTT by incubation for 30 min at 8°C on a rotating wheel. After centrifugation for 30 min at 200,000 *g*, the supernatant (containing the solubilized microsomes) was aliquoted and stored at –75°C for several weeks without loss of activity. For SDS gel separations, microsomes were solubilized in 50 mM potassium phosphate, pH 7.5, 0.5 mM EDTA, 0.5 mM DTT, 2% (w/v) digitonin, and 5% (v/v) glycerol. Microsomes were shaken for 90 min at 37°C followed by centrifugation for 30 min at 500,000 *g*.

### Fatty aldehyde dehydrogenase standard assay conditions

The reaction mixture with a total volume of 10  $\mu$ l contained 20 mM sodium pyrophosphate at pH 8.0, 1 mM NAD, 1% Triton X-100 (reduced form, v/v), and 50  $\mu$ M of the substrate pyrenedecanal (Ramidus, Lund, Sweden) added from a 2 mM stock solution in ethanol. The reaction was started by the addition of 2.5 ng to 3  $\mu$ g of protein sample. After 10–20 min at 37°C, the reaction was stopped with 30  $\mu$ l methanol. After 10 min centrifugation at 16,000 *g* and 4°C, the supernatant was used for HPLC analysis.

### Reversed phase HPLC analysis of reaction mixtures

The quantification of the substrate pyrenedecanal and the enzymatic product pyrenedecanoic acid was performed on an Agilent 1200 Series HPLC system using a Zorbax XDB-C8 USP-L7 column (Agilent Technologies, Vienna, Austria). Ten microliters of the sample were injected and eluted with 10 mM potassium phosphate buffer, pH 6.0, containing 81.25% (v/v) methanol at a flow rate of 1.0 ml/min for 8 min, followed by a gradient to 100% methanol at 8.5 min. At 12.5 min, the original mobile phase composition was restored within 30 s, resulting in a total run time of 13 min. Pyrenedecanoic acid was quantified by

fluorescence detection at 340-nm excitation and 400-nm emission and its identity confirmed by spiking with synthetic pyrenedecanoic acid.

### Culture of control and SLS patients' skin fibroblasts

All clinical investigations were approved by the Institutional Review Boards, according to the Helsinki Declaration, and performed after obtaining informed consent. Five fibroblast cultures were from the SLS cell collection of the Laboratory of Genetic Metabolic Diseases, Academic Medical Center at the University of Amsterdam, The Netherlands (7, 8). One fibroblast culture was from the Istituto Dermatologico dell'Immacolata-Istituto di Ricovero e Cura a Carattere Scientifico (IDI-IRCCS) Biochemistry Laboratory (9). All patients included in this study showed the typical signs and symptoms of SLS that had already been proven by measurement of fatty aldehyde dehydrogenase activity in fibroblasts and molecular analysis of the ALDH3A2 gene as indicated in Table 1. Human dermal fibroblasts from five healthy individuals were kindly provided by Christine Heufler (Department of Dermatology, Innsbruck Medical University). Informed consent and institutional approval of the studies were obtained at the respective institutions. Cells were grown in 75 cm<sup>2</sup> flasks at 37°C in an atmosphere of 5% CO<sub>2</sub> in DMEM (Gibco-21885; Invitrogen, Carlsbad, USA) containing GlutaMAX I, 1,000 mg/l D-glucose, and sodium pyruvate supplemented with 10% (v/v) fetal calf serum. Monolayers were collected by trypsinization and washed with Dulbecco's PBS buffer (Serva 47302). Cells were lysed by rapid freeze thawing in 0.1% (v/v) Triton X-100 (reduced form) in water containing 1% (v/v) of a protease inhibitor mixture (GE Healthcare 80-6501-23). The results shown were obtained with 0.8–3  $\mu$ g cellular protein per 10  $\mu$ l assay volume.

### Preparation of mouse tissues

All animal work was conducted in conformity with the Public Health Service Policy on Humane Care and Use of Laboratory Animals and approved by the respective committee of the Austrian Ministry of Science and Education. Fifteen selected tissues were extracted from five male and five female C57BL/6 mice (10 weeks old) and were immediately frozen in liquid nitrogen and stored at –75°C until analyzed. Depending on the weight of the tissue, they were homogenized in 200–500  $\mu$ l of preparation buffer using an Ultra-Turrax blade homogenizer (Ika, Stauffen, Germany) and centrifuged at 13,000 *g* for 10 min. The protein concentration in the supernatant was measured with the Bradford assay using BSA as standard. The samples were diluted in 100 mM Tris HCl, pH 8.5, to result in a protein amount of 1–2  $\mu$ g in 10  $\mu$ l assay volume, and fatty aldehyde dehydrogenase activity was measured as described above.

### Determination of the assay sensitivity using fibroblast protein

To determine the minimal amount of fibroblast protein required for the assay, we performed serial dilutions of control and SLS fibroblast protein. Setting the threshold to a signal-to-noise ratio >3, we found that 50 ng of control fibroblast protein was sufficient to determine the activity. For accurate determination of 5% residual activity in SLS patients' fibroblasts, 1  $\mu$ g protein was required.

### SDS gel electrophoresis and in-gel fatty aldehyde dehydrogenase activity determination

Proteins were separated on SDS polyacrylamide gels, stained with Coomassie Blue or silver according to standard procedures, and scanned using an ImageScanner (Amersham Biosciences). For quantification of fatty aldehyde dehydrogenase activity in gel

slices, the samples were applied to the gel in a modified sample buffer [60 mM Tris HCl, pH 6.8, 1% (v/v) glycerol, and 1% (w/v) SDS]. Thus, compared with standard SDS gels, mercaptoethanol, bromphenol blue, and the heating step were omitted. After electrophoresis in the cold room (gel temperature  $\sim 10^{\circ}\text{C}$ ), fatty aldehyde dehydrogenase was renaturated by washing the gel five times for 15 min in SDS-free buffer at  $4^{\circ}\text{C}$  [100 mM Tris HCl, pH 8.5, and 5% (v/v) glycerol]. The lanes were sliced into 18 fragments and incubated at  $37^{\circ}\text{C}$  on an orbital shaker in 40  $\mu\text{l}$  standard assay mixture containing 25% of the normal substrate concentration (12.5  $\mu\text{M}$  pyrenedecanal, in order to limit the amount of pyrenedecanal consumed for this experiment). The reaction was stopped after 10 min by the addition of 120  $\mu\text{l}$  methanol.

### Protein sequencing by ESI-MS

Gel slices were in-gel digested with trypsin and analyzed by nanospray mass spectrometry (LTQ Orbitrap XL; Thermo Finnigan) according to Ref. 10. Protein identification was performed using the SEQUEST algorithm in the Bioworks 3.3 software package (Thermo-Electron) and a rat database. The identified peptides were further evaluated using charge state versus cross-correlation number (Xcorr). The criteria for positive identification of peptides were  $X_{\text{corr}} > 1.5$  for singly charged ions,  $X_{\text{corr}} > 2.0$  for doubly charged ions, and  $X_{\text{corr}} > 2.5$  for triply charged ions.

### Cloning and purification of fatty aldehyde dehydrogenase, ALDH1A1, and ALDH2

Fatty aldehyde dehydrogenase cDNA was amplified from male Sprague Dawley rat liver cDNA by PCR and cloned into a Strep-tag fusion vector (pPR-IBA2 *Escherichia coli* vector, T7 promoter; IBA GmbH, Göttingen, Germany) using standard protocols. The protein was expressed in *E. coli* and purified according to standard protocols by Strep-tag affinity chromatography (IBA GmbH) and AMP-sepharose chromatography (AMP sepharose 4B; Sigma-Aldrich, Steinheim, Germany). Details will be published elsewhere. Recombinant human ALDH1A1 and ALDH2, prepared as detailed in Ref. 11, were kind gifts from Matteo Beretta and Bernd Mayer (Department of Pharmacology and Toxicology, University of Graz, Austria). All three enzymes were  $>95\%$  pure as judged by SDS gel electrophoresis.

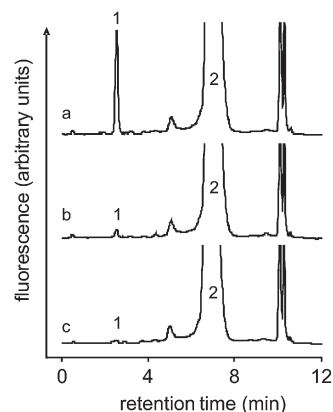
### Statistical analysis

All data are presented as means  $\pm$  SEM. Data were analyzed for statistical significance by Student's *t*-test using GraphPad Prism 5.01 (GraphPad Software, San Diego, CA). Values with  $P < 0.01$  were considered to be significantly different.

## RESULTS

### A novel assay to monitor fatty aldehyde dehydrogenase activity

In order to establish an assay for fatty aldehyde dehydrogenase activity that follows the formation of products and eventual by-products, we modified an existing HPLC assay for quantification of alkylglycerol monooxygenase (12). Pyrenedecanal, a fluorescently labeled aldehyde harboring a pyrene moiety at the distal end of the hydrocarbon chain, was used as substrate and formation of pyrenedecanoic acid served as readout. **Figure 1** shows separation of both product (peak 1) and substrate (peak 2) in the presence of control fibroblast extracts (trace a) and SLS



**Fig. 1.** Typical HPLC chromatograms of fatty aldehyde dehydrogenase determination in human fibroblast extracts. Trace a: A protein extract of fibroblasts of a healthy individual (2.9  $\mu\text{g}$ ) was incubated with pyrenedecanal in the presence of NAD for 10 min at  $37^{\circ}\text{C}$  in a total volume of 10  $\mu\text{l}$  (see Materials and Methods for details). The reaction was stopped by addition of 30  $\mu\text{l}$  of methanol, centrifuged, and 10  $\mu\text{l}$  injected into the HPLC system. Peak 1 represents the product pyrenedecanoic acid, and peak 2 is the remaining substrate pyrenedecanal. Trace b: Like trace a, but with a protein extract of fibroblasts of an SLS patient (2.7  $\mu\text{g}$ ) used instead of control fibroblast extract. Trace c: Chromatogram of the reaction mixture without protein.

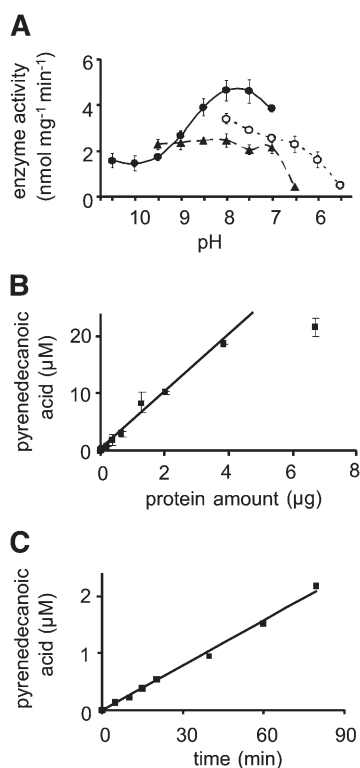
fibroblast extracts (trace b). Incubation without fibroblast protein did not yield any formation of pyrenedecanoic acid (Fig. 1, trace c). A high excess of pyrenedecanal was present at the end of the incubation period. No significant amounts of pyrenedecanoic acid ( $<10\%$  of pyrenedecanal) were formed. Pyrenedecanal (retention time 6.85 min) could be separated from pyrenedecanoic acid (retention time 7.44 min) with the HPLC conditions used (data not shown).

Pyrenedecanal was remarkably stable to chemical oxidation. Even in presence of a 1,000-fold excess of hydrogen peroxide, no formation of pyrenedecanoic acid was observed. Chemical oxidation of the substrate could only be achieved by addition of hydrogen peroxide together with sodium chlorite according to Ref. 13, which converted pyrenedecanal efficiently to pyrenedecanoic acid. No indication of polymerization of the aldehyde upon storage was observed (data not shown). No pyrenedecanoic acid formation was detected with heat-inactivated protein (5 min,  $95^{\circ}\text{C}$ ; data not shown). The cycle time was 13 min, and the detection limit was 10 fmol pyrenedecanoic acid.

In order to investigate the specificity of our novel assay for fatty aldehyde dehydrogenase compared with other aldehyde dehydrogenases, the activity of recombinant human ALDH1A1 [major substrate retinal (14)] and ALDH2 [major substrate acetaldehyde (14)] was tested in comparison to recombinant rat fatty aldehyde dehydrogenase [i.e., Aldh3a2, major substrate *cis,cis*-9,12-octadecadienal (15)]. Three dilution series (0.6–800 ng per 10  $\mu\text{l}$  assay volume) of the purified proteins were measured using our novel fatty aldehyde dehydrogenase assay. The results showed an aldehyde oxidizing activity of  $2.9 \pm 0.8 \text{ nmol}\cdot\text{mg}^{-1}\cdot\text{min}^{-1}$  for ALDH1A1 and  $3.5 \pm 0.4 \text{ nmol}\cdot\text{mg}^{-1}\cdot\text{min}^{-1}$  for ALDH2. These activities were about 10,000 times lower than the

enzymatic activity of purified recombinant fatty aldehyde dehydrogenase ( $36 \pm 9 \mu\text{mol}\cdot\text{mg}^{-1}\cdot\text{min}^{-1}$ ).

**Figure 2** shows dependence of the assay reaction on buffer system, protein concentration, and incubation time. Incubation of the reaction in Tris HCl buffer led to the formation of adducts with pyrenedecanal, resulting in an additional peak in the HPLC chromatogram and in a decreased amount of available free substrate. The additional peak was absent when Tris-free buffers were used. Tris HCl buffer did not show a detectable pH optimum in the range from 7.0–10.5. In contrast, both pyrophosphate buffer and potassium phosphate buffer yielded an optimum at pH 8.0 (Fig. 2A). Product formation increased linearly from 2.5 ng up to 2  $\mu\text{g}$  microsomal protein in 10  $\mu\text{l}$  assay volume and increased linearly up to 80 min (Fig. 2B, C, respectively). Although competition experiments of pyrenedecanal with dodecanal [ $K_m = 19 \mu\text{M}$  (15)] revealed comparable affinities of both aldehydes to fatty aldehyde dehydrogenase in rat liver microsomes, the reaction was not yet saturated at 50  $\mu\text{M}$  pyrenedecanal. This may explain the leveling off of the reaction at protein amounts of  $>4 \mu\text{g}$  protein (Fig. 2B).



**Fig. 2.** Dependence of fatty aldehyde dehydrogenase activity on incubation buffer, protein concentration, and incubation time. A: Enzymatic activity of fatty aldehyde dehydrogenase was measured in three buffer systems using pyrenedecanal as substrate and 20 ng rat liver microsomes (closed circles, sodium pyrophosphate buffer; open circles, potassium phosphate buffer; closed triangles, Tris HCl buffer) at pH values ranging from 5.5–10.5. B: Protein concentration dependence of fatty aldehyde dehydrogenase activity. Means  $\pm$  SEM of four independent series of measurement are shown. C: Incubation time dependence of the enzyme activity using 200 ng rat liver microsomes. One representative of three independent experiments is shown.

### Quantification of fatty aldehyde dehydrogenase activity in cultured SLS patient fibroblasts

We compared fatty aldehyde dehydrogenase activity in cultured fibroblasts of SLS patients versus healthy control cells (**Table 1**). Control cells displayed an activity of  $30.1 \pm 4.39 \text{ pmol}\cdot\text{mg}^{-1}\cdot\text{min}^{-1}$ . In SLS cells, fatty aldehyde dehydrogenase activity was decreased to  $3.8 \pm 0.36 \text{ pmol}\cdot\text{mg}^{-1}\cdot\text{min}^{-1}$ . This corresponds to a residual enzymatic activity of  $12.2 \pm 1.2\%$  in SLS patient fibroblasts. Due to the small incubation volume used in our assay (10  $\mu\text{l}$ ), we require only 1  $\mu\text{g}$  of SLS fibroblast protein for accurate determination. Other assays are typically performed with 10–50  $\mu\text{g}$  fibroblast protein in 200–500  $\mu\text{l}$  incubation volume (1, 5–7).

### Tissue distribution of fatty aldehyde dehydrogenase activity in mice

We determined fatty aldehyde dehydrogenase activity in selected tissues of 10 week old C57BL/6 mice using our novel assay with pyrenedecanal as substrate (**Table 2**). Activities were highest in liver, stomach, visceral fat, ovaries, and testes and lowest in heart and skeletal muscle. No significant sex difference in activity was found. To exclude the possibility of reaction quenching by endogenous unlabeled substrates or other interfering compounds, we spiked all reactions with rat liver microsomes displaying high fatty aldehyde dehydrogenase activity and found mean recovery of  $98 \pm 9\%$  in females and males (data not shown).

### Analysis of migration of fatty aldehyde dehydrogenase in a low-temperature SDS gel

We tested the migration properties of fatty aldehyde dehydrogenase in a low-temperature SDS gel. As shown in **Fig. 3**, the activity of the enzyme could be detected in gel slices, with a maximum in slice 7, which corresponds to a molecular mass of 95–110 kDa. In this slice, one major band was visible by Coomassie staining. ESI-MS showed fatty aldehyde dehydrogenase as most prominent protein, with 22 distinct, matching peptides resulting in 51.7% coverage of the protein sequence. Since the monomer mass of fatty aldehyde dehydrogenase is 55 kDa, these data are consistent with the hypothesis that fatty aldehyde dehydrogenase migrates as dimer in low-temperature SDS gels.

## DISCUSSION

The use of a pyrene fluorophore is a promising approach to label otherwise hardly detectable lipids for tracking with sensitive fluorescence-based methods. Many enzymes accept the pyrene label when positioned at the distal side of an aliphatic hydrocarbon chain with an optimal length of 10 to 12 carbon atoms (for review, see Ref. 16). The commercially available pyrenedecanal substrate was found to be well suited for monitoring of the fatty aldehyde dehydrogenase reaction and showed a clear preference for fatty aldehyde dehydrogenase that converted this aldehyde by four orders of magnitude better than two other aldehyde dehydrogenases tested. The reaction prod-

TABLE 1. Fatty aldehyde dehydrogenase activities in SLS patients' cells

Sample	n	Activity		ALDH3A2 Mutation			
		(pmol min <sup>-1</sup> mg <sup>-1</sup> )	% of Control	Allele 1 Nucleotide	Allele 1 Translation	Allele 2 Nucleotide	Allele 2 Translation
Controls 1–5 mean	16	30.1 ± 4.39	100 ± 14.6				
SLS patient 1	4	4.7 ± 0.62	15.5 ± 2.1	c.487_488insA	p.I163fs	c.487_488insA	p.I163fs
SLS patient 2	4	4.5 ± 0.58	14.9 ± 1.9	c.943C>T	p.P315S	c.1297_1298delGA	p.E433fs
SLS patient 3	4	4.3 ± 0.50	14.1 ± 1.7	c.1384_1387delGAAA	p.E462fs	c.1384_1387delGAAA	p.E462fs
SLS patient 4	4	3.1 ± 0.52	10.4 ± 1.7	c.178G>T	p.E60X	c.471delG	p.52-157del
SLS patient 5	4	4.4 ± 1.26	14.7 ± 4.2	c.901G>C, c.906delT	p.A301P, p.F302fs	c.901G>C, c.906delT	p.A301P, p.F302fs
SLS patient 6	3	1.2 ± 0.25	3.9 ± 0.83	c.1094C>T	p.S365L	c.471 + 2T>G	p.52-157del
SLS patients 1–6 mean	23	3.8 ± 0.36	12.2 ± 1.2				

Fatty aldehyde dehydrogenase was determined by formation of pyrenedecanoic acid from pyrenedecanal in homogenates of cultured fibroblasts as described in Materials and Methods. Values shown are means ± SEM. Numbers of nucleotides correspond to the coding region of the mRNA with the accession number NM\_000382.1 and numbers of protein residues to the protein with the accession number NP\_000373.1.

uct, pyrenedecanoic acid, can be quantified in nanomolar concentrations from microliter amounts of enzyme incubation mixtures, which contain microgram amounts of cellular or tissue protein. This assay is linear over a wide range of protein concentrations and incubation times and provides strongly improved sensitivity compared with assays based on monitoring of NADH generation (15). It appears more straightforward regarding substrate availability and sample handling than the assay used by James and Zoeller (1), which requires synthesis of [1-<sup>14</sup>C] hexadecanal from commercially available [1-<sup>14</sup>C] hexadecanol, lipid extraction of the reaction mixture, TLC, and X-ray film exposure. Other approaches require a more complex instrumentation, such as gas chromatography-mass spectrometry measurement of phytanic acid after hexane extraction (6) or quantification of 22-hydroxy-docosanoic acid after hexane extraction by ESI-MS (7). The values obtained for normal and SLS patients' fibroblasts with our novel assay are in good agreement with values obtained by other methods (6–8). Surprisingly, although the relative differences between normal and SLS fibroblasts compared

in the various studies published, several publications report about 300-fold higher activities in fibroblasts (1, 5, 9) using radioactive and fluorimetric methods. The reason for this discrepancy is not clear.

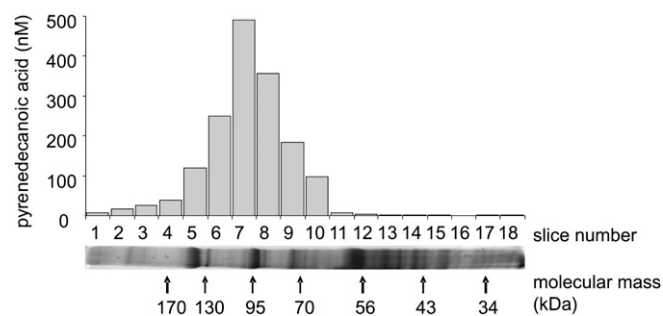
Fibroblasts also contain alcohol dehydrogenase activity (7), which might potentially consume the aldehyde substrate by reduction to the corresponding alcohol. However, in our incubations in presence of a high excess of NAD to achieve enzymatic oxidation of the aldehyde to the acid, <10% of the added pyrenedecanal was reduced to pyrenedecanol. This reduction would require NADH instead of NAD to proceed, which is only formed in small amounts (<0.1 μM) in fibroblast incubations by the fatty aldehyde dehydrogenase reaction. This is far below the *K<sub>m</sub>* of 36 μM of alcohol dehydrogenase for NADH (5).

The distribution of the enzymatic activity in mouse tissues presented here extends previous work (17) and correlates well with quantitative mRNA data available from databases obtained with the same mouse strain at similar age [(18); GEO accession number GSE9954]. Thus, activity of fatty aldehyde dehydrogenase in mouse tissues appears to be primarily determined by the relative amounts

TABLE 2. Tissue distribution of fatty aldehyde dehydrogenase activity in adult mice

Tissue	Female	Male
	Activity (pmol·mg <sup>-1</sup> ·min <sup>-1</sup> )	Activity (pmol·mg <sup>-1</sup> ·min <sup>-1</sup> )
Adrenal gland	331.2 ± 79.8	152.6 ± 41.1
Cerebellum	24.7 ± 4.4	14.9 ± 0.66
Cerebrum	59.3 ± 4.8	44.5 ± 2.8
Heart	13.0 ± 1.5	11.3 ± 0.58
Kidney	77.9 ± 14.8	122.4 ± 15.9
Large intestine	62.1 ± 8.7	69.7 ± 7.9
Liver	514.8 ± 65.4	343.9 ± 43.1
Lung	136.4 ± 19.3	129.0 ± 15.2
Ovaries	354.5 ± 30.1	–
Skeletal muscle	19.4 ± 2.1	13.8 ± 2.1
Spleen	90.5 ± 5.7	79.5 ± 12.9
Stomach	421.6 ± 43.8	484.3 ± 62.0
Subcutaneous fat	121.0 ± 17.7	89.6 ± 11.4
Testes	–	305.5 ± 33.0
Visceral fat	177.8 ± 18.9	309.8 ± 68.9

Fatty aldehyde dehydrogenase activity was determined in tissues of adult female and male C57BL/6 mice by the conversion of pyrenedecanal to pyrenedecanoic acid. Values shown are means ± SEM from measurement of individual tissues from five animals per group, except for ovaries and female lung, where only four tissues were analyzed.



**Fig. 3.** Determination of fatty aldehyde dehydrogenase activity in slices of low-temperature SDS gel slices. Digitonin-solubilized rat liver microsomes (150 μg) were separated on a 10% SDS gel at 10°C. The gel was washed five times for 15 min in SDS-free buffer. Fatty aldehyde dehydrogenase activity was measured in gel slices by the formation of pyrenedecanoic acid from pyrenedecanal. Slice 7 corresponded to a molecular mass of 95–110 kDa as calculated from migration of marker proteins. An additional parallel lane of solubilized microsomes stained with Coomassie brilliant blue is displayed beneath the curve.

of mRNA, rather than by translational or posttranslational regulatory mechanisms.

The power of our new method for determination of fatty aldehyde dehydrogenase is also demonstrated by its application to SDS gel slices. We performed this experiment to investigate whether fatty aldehyde dehydrogenase (Aldh3a2), like the aldehyde dehydrogenase Aldh3a1 (19), forms a dimer in the active state. For the digitonin-solubilized enzyme fatty aldehyde dehydrogenase, we could not obtain a homogenous molecular mass in gel filtration, but observed a broad spectrum of potentially aggregated protein complexes of high molecular weight (data not shown). We therefore decided to use low-temperature SDS gel electrophoresis, which had successfully been applied to quantify dimer versus monomer for nitric oxide synthase (20). We found a clear peak of activity in the gel slice corresponding to 95 to 110 kDa, which is twice the calculated monomeric mass of fatty aldehyde dehydrogenase of 55 kDa (Fig. 3). The broadening of the activity peak in comparison to the Coomassie band may be caused by protein diffusion in the washing step essential to remove the SDS. Since mass spectrometry confirmed fatty aldehyde dehydrogenase as the major protein component of the gel slice with maximal activity, we interpreted this as presence of a homodimer that is resistant to low-temperature SDS gel electrophoresis. ■■

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