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Saturation transfer difference NMR studies on substrates and inhibitors of succinic semialdehyde dehydrogenases

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ABSTRACT

Saturation transfer difference (STD) NMR experiments on *Escherichia coli* and *Drosophila melanogaster* succinic semialdehyde dehydrogenase (SSADH, EC1.2.1.24) suggest that only the aldehyde forms and not the *gem*-diol forms of the specific substrate succinic semialdehyde (SSA), of selected aldehyde substrates, and of the inhibitor 3-tolualdehyde bind to these enzymes. Site-directed mutagenesis of the active site cysteine311 to alanine in *D. melanogaster* SSADH leads to an inactive product binding both SSA aldehyde and *gem*-diol. Thus, the residue cysteine311 is crucial for their discrimination. STD experiments on SSADH and NAD^+/$NADP^+$ indicate differential affinity in agreement with the respective cosubstrate properties. Epitope mapping by STD points to a strong interaction of the NAD^+/$NADP^+$ adenine H2 proton with SSADH. Adenine H8, nicotinamide H2, H4, and H6 also show STD signals. Saturation transfer to the ribose moieties is limited to the anomeric protons of *E. coli* SSADH suggesting that the NAD^+/$NADP^+$ adenine and nicotinamide, but not the ribose moieties are important for the binding of the coenzymes.

Succinic semialdehyde dehydrogenases (EC1.2.1.24, SSADH) belong to the superfamily of aldehyde dehydrogenases [1] and catalyze the NAD^+ or $NADP^+$ dependent oxidation of succinic semialdehyde (SSA) to succinic acid. SSADH has been identified in a variety of prokaryotic and eukaryotic organisms. Its known functions include a role in the utilization of exogenous GABA or endogenous 4-hydroxybutyric acid as carbon and energy sources in bacteria [2,3]. In plants [4] and some vertebrate tissues, this enzyme participates in an alternative 2-ketoglutarate degradation pathway, the GABA shunt. Furthermore SSADH is crucial for the degradation of the inhibitory neurotransmitter GABA in the nervous system of vertebrates [5,6]. The study of SSADH in invertebrates is limited so far to an enzyme from lobster [7] and to *Drosophila (D.) melanogaster* [8]. Plants require a functional SSADH to resist oxidative stress [4]. In humans SSADH deficiency has recently been recognized as a cause for severe hereditary neurological conditions which illustrates its crucial function in brain metabolism [5,6]. Despite the growing recognition of the importance of SSADH for organisms of several phyla, no structural information, such as X-ray crystallography or nuclear magnetic resonance (NMR) data, has been reported, and no information is available on the specific binding characteristics of substrates, cosubstrates or other ligands to this enzyme.

Among the variety of NMR experiments available, saturation transfer difference (STD) spectroscopy is an excellent tool for the investigation of protein–ligand interactions and the characterization of binding epitopes without the prerequisite of protein isotope labeling [9–11].

In this study, we have overexpressed the gene encoding *Escherichia* (*E.*) *coli* SSADH and characterized the biochemical properties of the purified enzyme. Together with this prokaryotic SSADH we have studied a recombinant form of the eukaryotic homolog, *D. melanogaster* SSADH, with NMR spectroscopy. STD NMR experiments on the SSADH enzymes in combination with the specific substrate SSA, other aldehydes, and the NAD^+/$NADP^+$ coenzymes were performed to explore the aldehyde substrate form specificity and to map the binding epitopes of the coenzymes.

Materials and methods

Bacterial strains, plasmids, chemicals, and column materials. Bacterial cultures were grown in Luria–Bertani (LB) medium. Polymerase chain reaction (PCR) products were cloned into pCR2.1-Topo and introduced into *E. coli* Top10 cells (Invitrogen). The hexa-His (His_6) tag expression vector pQE30 and its *E. coli* host strain M15 as well as Ni^{2+}–NTA agarose were purchased from Qiagen. SSA, NAD^+, and $NADP^+$ were obtained from Sigma, benzaldehyde, 3-tolualdehyde, 3-nitrobenzaldehyde, 3-, and 4-carboxybenzaldehyde from Aldrich.

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Recombinant DNA techniques. DNA from *E. coli* DH5α was purified as outlined in [12]. The *E. coli* SSADH gene was isolated from *E. coli* DH5α genomic DNA by PCR amplification (Expand high fidelity PCR kit, Roche) using the primer CCCGGGATCCATGAAACTTAAC GACAGTAACTTATTCC and CCCGGGAAGCTTAAAGACCGATGCAC ATATATTTGATTTC designed from the GenBank sequence M88334 [13]. The PCR product was ligated into BamHI/HindIII-cut pQE30 yielding pQE-EcSSADH, and transformed into *E. coli* M15.

Protein expression in E. coli, protein purification, and enzymatic assays. EcSSADH expression was induced in exponentially growing *E. coli* M15 carrying pQE-EcSSADH for 3 h by the addition of 1 mM isopropyl thio-β-D-galactoside. Purification of His_6-EcSSADH was performed by native Ni^{2+}–NTA agarose chromatography (Qiagen). MalE-DmSSADH wild type (WT) and mutated forms were prepared as outlined in [8]. Protein concentration was determined by the Coomassie blue dye binding method [14]. For calculations of enzyme molarities a molecular mass of 53,000 Da for His_6-EcSSADH and 98,000 Da for MalE-DmSSADH was used. Enzyme assays were performed at 22 °C in 1 ml volumes of 100 mM Na_2HPO_4/NaH_2PO_4 buffer (NaP_i), pH 6–10, using variable concentrations of NAD^+/$NADP^+$ and SSA or other aldehyde substrates. A molar absorption coefficient of 6220 M^{-1} cm^{-1} was used in calculations for NADH and NADPH production. One unit (U) is defined as the amount of enzyme converting 1 μMol aldehyde substrate per minute. The Michaelis–Menten constants (K_m) of the enzymes were determined at pH 8.5 using 0.5–1 μg of Ec-His_6-SSADH. A standard assay mixture contained 100 mM NaP_i, pH 8.5, 1 mM $NADP^+$, and 50 μM SSA or 1 mM for all other aldehyde substrates used in this study.

NMR spectroscopy studies. For NMR experiments, NaH_2PO_4/Na_2HPO_4, pH 7.4 (NaP_i) buffer solution was prepared using D_2O (Aldrich) instead of H_2O. The NMR samples were prepared by addition of educt, cosubstrates, and product solutions in D_2O/NaP_i buffer to stock solutions of the protein in D_2O/NaP_i buffer. Protein stock solutions varied from 15 μM to 150 μM. Aromatic and aliphatic aldehydes were dissolved in DMSO-d_6 from Sigma–Aldrich and mixed with the protein stock solutions, such that the DMSO-d_6 content did not exceed 5% (v/v) in the sample. All NMR measurements were performed at 400 MHz on a Bruker DRX 400 spectrometer at 298 K using a 5 mm BBO probehead equipped with z-gradient and a GREAT 1/10 gradient amplifier. Proton NMR experiments were recorded using an excitation sculpting pulse scheme [15] for water suppression. Saturation transfer difference spectra with WATERGATE scheme were recorded according to a published report [10]. Typical experimental parameters were: selective presaturation of the protein was achieved by a train of 50–100 Gauss-shaped pulses of 50 ms with a strength of $(\gamma/2\pi)B_1$ = 26 Hz. The on-resonance irradiation of the protein was performed at 0.69 ppm for SSADH from *E. coli* and 0.78 ppm from *D. melanogaster*. Off-resonance irradiation was applied at 20 ppm, where no protein signals were present. The usual relaxation delay was set to 2 s. To eliminate protein resonances from the spectrum, a spin-lock filter ($T_{1\rho}$-filter) was used. The total number of scans amounted to 256 or 512 according to the expected signal strength. A spectral width of 12 ppm was typically chosen. The spectra were processed by multiplication with an exponential line broadening function of 0.3–1 Hz prior to Fourier transformation. The data were processed and analyzed with Bruker Topspin 1.2 software.

Results

Cloning, overexpression, and characterization of E. coli SSADH

The *E. coli* gene homolog (M88334) of mammalian SSADH (AK052703) and *D. melanogaster* SSADH [8] was amplified by PCR from *E. coli* DNA, cloned into pQE30, and the encoded protein overexpressed in *E. coli* M15. Abundant expression of His_6-EcSSADH was observed (Fig. 1A, lane 1). His_6-EcSSADH was purified from the soluble fraction (Fig. 1A, lane 2) of a lysate by Ni^{2+}–NTA agarose chromatography with yields of 4–5 mg/100 ml culture (Fig. 1A, lanes 3 and 4). The recombinant product was enzymatically active with SSA as aldehyde substrate and $NADP^+$ as cosubstrate (Fig. 1B), with a typical specific activity of ~3–4 U/mg. NAD^+ also acted as cosubstrate, but the reaction rates were more than 20-fold lower than those with $NADP^+$ (Fig. 1B). His_6-EcSSADH showed a broad pH-optimum of $NADP^+$-dependent SSA oxidation activity centered around pH 8.5 with approximately 75% activity at pH 7.4 (not shown). The determination of substrate K_M values at pH 8.5 yielded 7.8 μM (±1.2 μM, $n = 5$) for SSA and 44.6 μM (±6 μM, $n = 3$) for $NADP^+$. Apart from its strong activity towards SSA, His_6-EcSSADH also oxidizes some long chain linear alkanals, such as *n*-butanal, *n*-pentanal, and *n*-hexanal (not shown), as well as some aromatic aldehydes like benzaldehyde, 3-carboxybenzaldehyde, 4-carboxybenzaldehyde, 3-nitrobenzaldehyde, albeit with lower catalytic efficiency compared to SSA (Fig. 1C). 3-Tolualdehyde did not possess substrate properties, but proved to be a potent inhibitor of SSA oxidation, with an estimated IC_{50} of ~80 μM (Fig. 1D).

Binding of succinic semialdehyde (SSA) to E. coli and D. melanogaster SSADH

While aliphatic and aromatic aldehydes exist in aqueous solutions as equilibrium between the aldehyde and the *gem*-diol form (Fig. 1E, 1 and 2), the formation of a lactone (Fig. 1E, 3) is also conceivable in the case of SSA [16,17]. ^1H NMR experiments with 1 mM SSA in NaP_i, pH 7.4 (90% D_2O) suggest that the acyclic molecular species (Fig. 1E, 1 and 2) are present at a ratio of 1:1. In the ^1H NMR spectra (not shown), the three resonances at 9.56 ppm (s, 1H, A), 2.67 ppm (dt, 1.2 Hz, 6.8 Hz, 2H, M), and 2.43 ppm (t, 6.8 Hz, 2H, X) are attributed to the aldehyde form. The absorptions at 4.96 ppm (t, 5.6 Hz, 1H, A), 1.78 (dt, 5.6 Hz, 8.2 Hz, 2H, M), and 2.21 ppm (t, 7.7 Hz, 2H, X) are interpreted accordingly as arising from the *gem*-diol form. For the lactone a more complex spectrum would be expected, therefore it is concluded that the lactone (Fig. 1E, 3) is not observed under the experimental conditions used. When SSA in solution is subjected to a ^1H NMR STD experiment in the presence of *E. coli* His_6-SSADH, only resonances of the aldehyde form are observed (Fig. 2A). Since magnetization transfer in such experiment proceeds from the protein to the small molecule ligand and is detected as enhanced signals of the ligand after its dissociation from the enzyme [10], this observation strongly suggests that only the aldehyde form of SSA binds to the *E. coli* enzyme. We extended this observation to a eukaryotic SSADH, NAD^+-dependent *D. melanogaster* SSADH. The enzyme was available from a previous study as enzymatically active maltose binding fusion protein (MalE-DmSSADH, [8]). The STD experiment with MalE-DmSSADH in the presence of its substrate SSA exhibited again only resonances of the aldehyde form, while the *gem*-diol form was not observed (Fig. 2B).

We recently generated a *D. melanogaster* MalE-SSADH variant by site-directed mutagenesis, where cysteine (Cys) 311 was mutated to alanine (Ala). This cysteine residue was considered the most likely candidate for the catalytically active nucleophile involved in the formation of the thiohemiacetal intermediate. Indeed, the Cys311Ala mutant protein was still soluble but unable to catalyze SSA oxidation [8]. By means of STD experiments, both NAD^+ (not shown) and SSA binding to mutated MalE-DmSSADH was demonstrated (Fig. 2C), which provided additional evidence for the proper folding of the mutated enzyme. Remarkably, in contrast to the wild type MalE-DmSSADH, the Cys311Ala mutant showed signals from both the aldehyde and the *gem*-diol form of SSA in STD experiments (Fig. 2C). This suggested that Cys311 is