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L-Cystine inhibits aspartate-β-semialdehyde dehydrogenase by covalently binding to the essential ^{135}Cys of the enzyme

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Abstract

Aspartate-β-semialdehyde dehydrogenase (ASADH) from *Escherichia coli* is inhibited by L- and D-cystine, and by other cystine derivatives. Enzyme inhibition is quantitatively reversed by addition of dithiothreitol (DTT), dithioerythrytol, β-mercaptoethanol, dimercaptopropanol or glutathione to the cystine-inactivated enzyme. Cystine labeling of the enzyme is a pH dependent process and is optimal at pH values ranging from 7.0 to 7.5. Both the cysteine incorporation profile and the inactivation curve of the enzyme as a function of pH suggest that a group(s) with pK_a of 8.5 could be involved in cystine binding. Stoichiometry of the inactivation reaction indicates that one cysteine residue from the enzyme subunit is reactive against cystine, as found by direct incorporation of radioactive cystine into the enzyme and by free-thiol titration of the enzyme with 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) before and after the cystine treatment. One mole of cysteine is released from each mol of cystine after reaction with the enzyme. ASA, NADP and NADPH did not prevent cystine inhibition. The [^{35}S]cysteine-labelled enzyme can be visualized after electrophoresis in polyacrylamide gels and further detection by autoradiography. After pepsin treatment of the [^{35}S]cysteine-inactivated enzyme, a main radioactive peptide was isolated by HPLC. The amino acid sequence of this peptide was determined as FVGGN(Cys)$_2$TVSL, thus demonstrating that the essential ^{135}Cys is the amino acid residue modified by the treatment with cystine.

Keywords: Cystine; ASADH inhibition; Covalent thiol modification

1. Introduction

L-Aspartate-β-semialdehyde dehydrogenase (ASADH; E.C.1.2.1.11) from *Escherichia coli* is a dimeric enzyme composed of identical subunits. In the biosynthetic direction ASADH catalyzes the formation of L-aspartate-β-semialdehyde (ASA) by the reductive dephosphorylation of L-β-aspartyl-phosphate utilizing NADPH [1,2]. The ASADH reaction is related to the reaction catalyzed by glyceraldehyde-3-phosphate dehydrogenase and similar chemical mechanisms have been proposed for each enzyme [3,4]. The suggested mechanism involves the formation of a thioester intermediate resulting from attack of a cysteine thiolate on the substrate carbonyl group, followed by hydride transfer to NADP. Subsequent attack on the thioester intermediate by an oxygen anion of bound inorganic phosphate leads to expulsion of the cysteine thiolate and formation of the phosphorylated product. Substrate protection studies against *N*-ethylmaleimide inactivation performed with ASA have suggested that the essential cysteine is located in or near the enzyme active site. In addition, pH studies have indicated a role for a neutral acid group that must be ionized for enzymatic activity. The pK_a value determined for this group is similar to that for the enzymatic group that leads to loss of enzyme activity when modified by *N*-ethylmaleimide [5]. The substrate-binding site of aspartate semialdehyde dehydrogenase from *E. coli* has also been studied by affinity labeling with L-2-amino-4-oxo-5-chloropentanoic acid. The substrate analogue irreversibly inactivates the enzyme with a pseudo-first-order kinetics and with a half-of-the-sites reactivity. The aspartate semialdehyde substrate protects the enzyme against the

Abbreviations: ASA, aspartate-β-semialdehyde; ASADH, aspartate-β-semialdehyde dehydrogenase; DTT, dithiothreitol; DTNB, 5,5′-dithiobis-2-nitrobenzoic acid; NADPH, β-nicotinamide adenine dinucleotide phosphate, reduced form

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inactivation. A single group is labeled at the active site and is concluded to be the side-chain of a histidine residue [6,7].

The enzyme from *E. coli* contains four cysteine residues [8] and has been studied by oligonucleotide-directed mutagenesis. The focus on this investigation was to examine the role of a cysteine residue that had been previously identified by chemical modification with an active site directed reagent. Substitution of this cysteine at position 135 with an alanine results in complete loss of enzyme activity. However, changing this cysteine to a serine yields a mutant enzyme with a maximum velocity that is 0.3% that of the native enzyme. This C135S mutant has retained essentially the same affinity for substrates as the native enzyme, and the same overall conformation as reflected on gel electrophoresis and in identical fluorescence spectra. The pH profile of the native enzyme shows a loss in catalytic activity upon protonation of a group of pK_a value of 7.7. The same activity loss is observed at this pH with the serine-135 mutant, despite the differences in the pK_a values for a cysteine sulfhydryl and a serine hydroxyl group that have been measured in model compounds. This observed pK_a value may reflect the protonation of an auxiliary catalyst that enhances the reactivity of the active site cysteine nucleophile in the native ASADH [9].

In this paper we describe the inhibition of the ASADH from *E. coli* by L-cystine and by several cystine derivatives, and we demonstrate that this inhibition is produced as a result of the covalent binding of a cysteine moiety from the cystine and the ^{135}Cys of the enzyme.

2. Materials and methods

2.1. Materials

Electrophoretically pure ASADH from *E. coli* was purified using previously published methods [6]. ASA was provided by SB Medicinal Chemistry and stored desiccated at −20 °C. Bis-*N*-acetyl-cystine was prepared by incubating 10 mM *N*-acetyl-cysteine in the presence of 50% dimethylsulfoxide [10]. Pre-stained molecular weight markers were purchased from Bio-Rad. L-[^{35}S]Cystine (5 GBq/mmol) was purchased from Amersham. Pepsin, trypsin, V8 protease, 2,2-dithiodipyridine, 4,4-dithiodipyridine, L- and D-cysteine, oxidized and reduced Coenzyme A, L- and D-cystine, oxidized and reduced glutathione, oxidized and reduced dithiothreitol (DTT), *N*-acetyl-L-cysteine, disulfiram and 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) were purchased from Sigma.

2.2. Enzyme assays

Determination of initial rates of ASADH activity was based on the increase in absorbance at 340 nm due to the production of NADPH. Readouts were performed on a Molecular Devices spectrophotometer equipped with a thermostatic cell holder. Assays were conducted for 1 min at 30 °C in a total volume of 200 μl. Typical assays contained 150 mM HEPES (pH 7.5), 10 mM potassium phosphate buffer (pH 7.5), 0.5 mM ASA, 0.5 mM NADP and 0.12 μM (subunit concentration) ASADH. Enzyme activity was routinely assayed in the phosphorylating direction with ASA as the substrate. A mixture of ASA and NADP was added last to assay mixtures to initiate the reactions. The effect of several thiol-containing compounds on ASADH activity was tested by pre-incubating each compound with the enzyme in reaction buffer for 15 min at room temperature. After this time, reaction was started by adding a mixture of substrates ASA and NADP.

The effect of L- and D-cystine, and the other cystine derivatives on ASADH activity was tested by incubating the enzyme (1 μM in subunit concentration) in 50 mM HEPES, pH 7.5, for 10 min at 21 °C in the presence of the compounds to be tested. L-Cystine was added at final concentrations between 1 nM and 20 μM, whereas D-cystine was tested at concentrations between 50 nM and 1 mM. Fresh aqueous solutions of the amino acids were used for each set of experiments. At fixed times, aliquots were removed and assayed for ASADH activity. Reversibility of the inhibition produced by these compounds was determined by removal of the excess of inhibitor by centrifuging reaction samples in Ultrafree-MC 5,000 filters (cut-off 5000 Da) and successive washing with reaction buffer. After extensive washing, DTT was added to the reaction mixture at a 1 mM final concentration and incubated for 10 min at room temperature. After this time, enzyme activity present in the samples was tested again as described above.

2.3. Determination of thiol groups

Thiol groups in native and in denatured enzyme were determined at room temperature with 0.5 mM DTNB using 0.1 M Tris–HCl, pH 8. Denaturating agents were 1% sodium dodecyl sulfate or 8 M urea [11].

2.4. Labeling of ASADH with L-[^{35}S]cystine

Experiments on labeling of ASADH enzyme with L-[^{35}S]cystine were done as follows. Assays were conducted in 96-well microtiter plates for 15 min at 21 °C in a total volume of 100 μl. Typical reaction mixtures contained 150 mM HEPES (pH 7.5), 10 mM potassium phosphate buffer (pH 7.5), L-[^{35}S]cystine and 1.4 μM ASADH. After reaction, 200 μl of 5% trichloroacetic acid was added to each well and the plates were allowed to stand for 10 min at room temperature. The content of the well was transferred to a glass-fiber filter plate (Multiscreen-FC, Millipore) and washed three times with 200 μl of 5% trichloroacetic acid and two times with 200 μl of ethanol. Microplates were allowed to dry for 15–30 min at 60 °C, then 25 μl of Microscint-0 was added to each well and radioactivity was determined in a Top-Count scintillation counter (Packard

Instruments). All the results were corrected for counting efficiency and for isotopic decay.

For evaluation of the ASADH activity of the enzyme labeled with [^{35}S]cystine, samples of the enzyme were treated as described above and aliquots were taken and the protein was separated from the excess of free label. Separation of labeled ASADH and excess of L-[^{35}S]cystine was achieved by centrifuging reaction samples in Ultrafree-MC 5000 filters and successive washing with reaction buffer. After this step, the enzyme activity was determined as described above.

2.5. Detection of [^{35}S]cystine-labeled ASADH in polyacrylamide gels

Detection of [^{35}S]cystine-labeled ASADH in polyacrylamide gels was performed by using a TCA-precipitated reaction mixture obtained according to the above protocol. After centrifugation, the protein was washed twice with methanol, dried and re-dissolved in 100 μl of water. Different amounts of protein were subjected to polyacrylamide gel electrophoresis in the presence of SDS, essentially as described by Laemmli [12] by using 12% polyacrylamide gels. The sample loading buffer used in these experiments did not contain β-mercaptoethanol. As molecular weight markers, we used the following pre-stained standards: lysozyme, soybean trypsin inhibitor, carbonic anhydrase, ovalbumin, bovine serum albumin, phosphorylase B and β-galactosidase. After electroforesis, the gel was vacuum-dried and radioactivity present was detected by exposure to a Hyperfilm-MP (Amersham) for 10 days.

2.6. Characterization of [^{35}S]cysteine as a reaction product

Detection of free [^{35}S]cysteine released as a consequence of the reaction between [^{35}S]cystine and ASADH was done by subjecting reaction samples to thin-layer chromatography. The labeling reaction, as described above, was transferred to an Ultrafree-MC 5000 cutoff filter and centrifuged for 5 min at 3,000 × g. The filtrate was concentrated to dryness in a Savant rotating evaporator, re-dissolved in 10 μl of water and applied to a Silicagel F_{254} plate (Merck). Development was performed in ethanol/water (70:30, v/v). After chromatography, the cysteine spots in the plate were detected by spraying a control sample with ninhydrin, scrapped off, added of liquid scintillator and counted in a Tri-Carb liquid scintillation counter (Packard Instruments). Appropriate controls were done in order to discard cross-contamination between the spots corresponding to cystine and cysteine.

The high molecular weight fraction from the filtration step was precipitated with trichloroacetic acid and the bound radioactivity determined by liquid scintillation counting. In order to determine if the radioactivity bound to the protein would be released by DTT, the precipitated protein was collected by centrifugation, washed (three cycles with 1% trichloroacetic acid and two cycles with ethanol), vacuum-dried, re-suspended in 10 mM Tris–HCl, pH 8.0 and added of 1 mM DTT for 10 min at room temperature. The enzyme was precipitated again, washed and counted.

2.7. Protease treatment and amino acid sequence

For large-scale labeling, 2 mg of ASADH was incubated with L-[^{35}S]cystine in the conditions described above for the small-scale reactions. The labeled protein was digested with pepsin in 5% formic acid using a protease/ASADH concentration ratio of 1:50. Peptides were separated in a LC-318 Supelcosil HPLC column (4.5 by 250 mm). The elution was performed isocratically at a flow rate of 1 ml/min. The mobile phase contained a mixture of acetonitrile/water (15:85, v/v) containing 0.1% trifluoroacetic acid. A major radioactive peptide was collected and its sequence was determined using an Applied Biosystems Procise Sequencer.

2.8. Mass spectrometry analysis

An ion-trap mass LCQ spectrometer (Finnigan, ThermoQuest, USA) with an electrospray interface was used in this work. The standard front end of the electrospray probe originally included in the LCQ package was removed and substituted by miniaturized spraying devices mounted onto a manipulator (Zeiss) whose position in front of the heated capillary was monitored using a stereo microscope. Ionization was performed with no sheath gas assistance (pure electrospray). Off-line nanospray ionization [13] was carried out using disposable gold-coated capillary probes (The Protein Analysis Company, Denmark), and a microinjector air pressure device (Eppendorff). Position of the probe, electrospray voltages and typical flows were similar to those described by Shevchenko et al. [13].

3. Results and discussion

3.1. Effect of different thiol compounds on ASADH activity

Although interaction of ASADH and other aldehyde dehydrogenases with several thiol-containing compounds and thiol-modifying reagents has been described and characterized in detail in previous reports [4,5,14], no information has been published about the possible interaction between ASADH and cysteine derivatives. In a preliminary test, several thiol-reacting compounds were preliminarily tested as inhibitors of ASADH when added to the reaction mixture at a 100 μM concentration. Compounds were pre-incubated with the enzyme in reaction buffer in the conditions described above. Tested compounds were 2,2-dithiodipyridine, 4,4-dithiodipyridine, L- and D-cysteine, oxidized and reduced Coenzyme A, L- and

D-cystine, oxidized and reduced glutathione, oxidized and reduced DTT, *N*-acetyl-L-cysteine, disulfiram and DTNB. Disulfiram, 2,2-dithiodipyridine and 4,4-dithiodipyridine have been reported as inhibitors of the cytoplasmic and mitochondrial aldehyde dehydrogenases from sheep liver [3]. Amongst all of them, only L- and D-cystine, DTNB and oxidized glutathione produced significant inhibition (100%, 98%, 100% and 65%, respectively) of the enzyme activity. Aliquots from the enzyme samples treated with these reagents were removed of excess of reagent and treated with 1 mM DTT for 10 min. After this time all the inactivated samples recovered 90–100% of their initial activity, thus likely indicating the presence of a disulfide bridge between the inhibitor and the enzyme molecule, which is cleaved by the action of the DTT. This reactivating effect was also found when the inactivated enzyme was treated with other thiols such as dithioerythrytol, β-mercaptoethanol, dimercaptopropanol or reduced glutathione at 1 mM concentration.

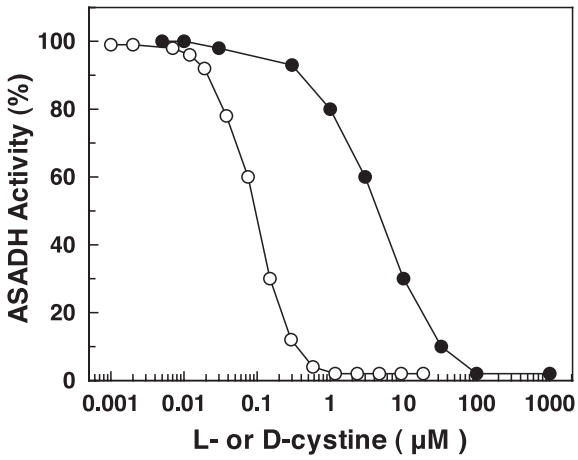

Fig. 1. Effect of different concentrations of L- (○) and D-cystine (●) on ASADH activity. The enzyme (0.12 μM, subunit concentration) was incubated with the two compounds at pH 7.5, and the enzyme activity was measured as described in Materials and methods.

3.2. ASADH inhibition by cystine and cystine derivatives

A more detailed study on the effect of cystine was carried out. In a preliminary step, L- and D-cystine, *N*,*N*′-diacetyl-L-cystine, L-cystine hydroxamate, L-cystine dimethyl ester, L-cystine diethyl ester and L-cystine di-β-naphthylamide were tested as ASADH inhibitors at 10 μM concentration in the assay conditions reported above (Table 1). *N*,*N*′-Diacetyl-L-cystine and L-cystine di-β-naphthylamide were inactive as ASADH inhibitors at this concentration. L-Cystine hydroxamate acted as a very weak inhibitor, producing a 20% inhibition of the enzyme activity. L-Cystine, D-cystine, L-cystine dimethyl ester and L-cystine diethyl ester were more potent enzyme inhibitors. These differences could probably be due to the important influence of the polar environment of the protein on the interaction with the cystine derivatives more than to differences in the chemical reactivity of the tested cystine derivatives. Aliquots of the enzyme-inactivated samples were removed of excess of reagent and treated with 1 mM DTT as described above. All the inactivated samples recovered 90–100% of their initial activity. As in the previous case, this reactivating effect was also found when the inactivated enzyme was treated with either dithioerythrytol, β-mercaptoethanol, dimercaptopropanol or reduced glutathione at 1 mM concentration.

Table 1
Effect of different cystine derivatives on ASADH activity at a 10 μM concentration and reactivation by 1 mM DTT of the inactivated enzyme

Compound	Inhibition (%)	Reactivation by DTT
L-Cystine	100	yes
D-Cystine	70	yes
N,*N*′-diacetyl-L-cystine	0	–
L-Cystine hydroxamate	20	yes
L-Cystine dimethyl ester	67	yes
L-Cystine diethyl ester	68	yes
L-Cystine di-β-naphthylamide	0	–

The enzyme (0.12 μM, subunit concentration) was incubated with the compounds at pH 7.5, and the enzyme activity was measured as described in Materials and methods.

Although both cystine isomers acted as inhibitors of ASADH activity, we found important differences in their comparative potency (see Fig. 1). L-Cystine was found to act as a much more potent inactivator of ASADH activity than the D-cystine. The concentration of the inhibitor producing a 50% inhibition found for the L-isomer (0.1 μM) is considerably lower than the value found for the D-amino acid (4 μM). This effect can be explained taking into consideration the conformational environment of the protein around the site of attack by the cystine.

3.3. Labeling of ASADH with [^{35}S]cystine: characterization of [^{35}S]cysteine as a reaction product

In order to better characterize the interaction between L-cystine and ASADH, we tried to identify the products from the reaction between L-cystine and ASADH. By using thin-layer chromatography of these samples, we detected the presence of free [^{35}S]cysteine after reaction between [^{35}S]cystine and ASADH. Furthermore, the high molecular weight fraction from the filtration step was precipitated with trichloroacetic acid and the bound radioactivity determined by liquid scintillation counting. Interestingly, the amount of radioactivity present in the precipitated enzyme was similar to the radioactivity found in the cysteine fraction isolated from the TLC plates. The radioactivity bound to the protein was quantitatively released by DTT treatment and less than 1% of the initial radioactivity was found in the re-precipitated protein. The radioactive compound released by the action of DTT was identified as the amino acid cysteine by subjecting a sample to thin-layer chromatography in the conditions described above. These facts allowed us to establish that reaction between cystine and ASADH con-

sisted on the binding to the enzyme of a cysteine moiety from the cystine and the subsequent release of the other cysteine moiety.

Binding of [^{35}S]cystine to ASADH was dependent on the amount of enzyme in the assay mixture. Fig. 2 depicts the correlation between label incorporated into precipitated protein and the amount of ASADH added to the reaction. Hypothetically assuming that 1 mol of cystine renders 1 mol of enzyme-bound cysteine, from Fig. 2 we can estimate a ratio of 2 mol of cysteine bound per mole of enzyme. The same linear correlation can be established for the amount of ASADH present in the assay and the amount of [^{35}S]cysteine released from the reaction (not shown). In both cases, the ratio between released [^{35}S]cysteine and labeled enzyme was 2 mol of amino acid per mole of enzyme.

Labeling of ASADH by L-[^{35}S]cystine was a time-dependent process. The labeling reaction was very rapid, as it occurs with the L-2-amino-4-oxo-5-chloropentanoic acid, the inactivator used by Biellman et al. [6] in their studies on characterization of essential residues in ASADH. The enzymatic activity of the ASADH enzyme treated with cystine decreased in proportion to the label incorporated into the enzyme molecule. Fig. 3 depicts the correlation between the ASADH activity and the average number of cysteine molecules incorporated per enzyme molecule. In the absence of incorporation, the enzyme displayed a 100% activity, whereas incorporation of two cysteines per enzyme molecule led to a complete inactivation of the enzyme.

Thiol titration of ASADH with DTNB indicated the presence of three reactive groups per subunit, according to the results obtained by Biellman et al. [15]. Titration of the cystine-inactivated enzyme revealed only two reactive thiols per subunit, suggesting that reaction between cystine and ASADH leads to the formation of a disulfide bridge between one cysteine from the enzyme and one from cystine.

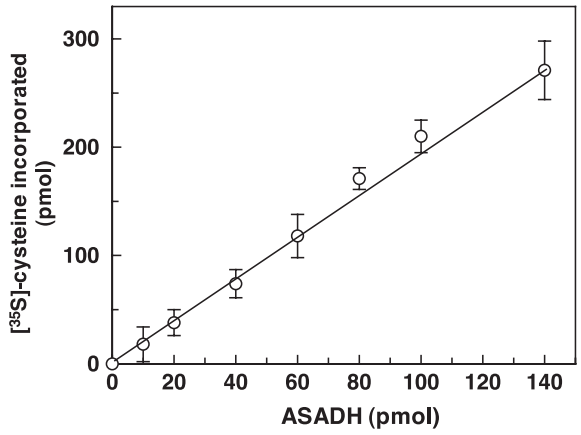

Fig. 2. Effect of protein concentration on the ASADH labeling by [^{35}S]cystine at pH 7.5. [^{35}S]cystine was calculated assuming that from each reacting cystine a cysteine moiety remains bound to the enzyme).

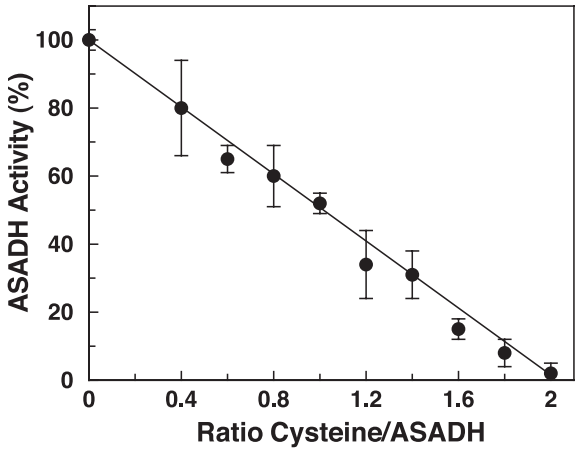

Fig. 3. Stoichiometry of [^{35}S]cystine incorporation by ASADH. The enzyme was incubated at pH 7.5 in the presence of the inactivator at 4 °C in the conditions described above. Aliquots were removed at the indicated time intervals and assayed for enzyme activity and for incorporation of radioactive cystine as described in Materials and methods.

3.4. pH dependence of the reaction between cystine and ASADH

The binding of L-[^{35}S]cystine to the enzyme was determined in a pH range of 7.0 to 10.0, using 50 mM potassium phosphate buffer. Fig. 4 depicts the evolution with the pH of the average number of cysteines incorporated per mole of enzyme. The binding curve showed that the optimal pH for binding was 7.0 to 7.5, and that a residue with an apparent pK_a of 8.5 could be involved in binding. A likely candidate for such a group is the SH-group of cysteine in solution. This result is similar to that found by Shevchenko et al. [13] when monitoring the effect of pH on the incorporation of L-[^{35}S]cysteine into the rabbit muscle glyceraldehyde-3-phosphate dehydroge-

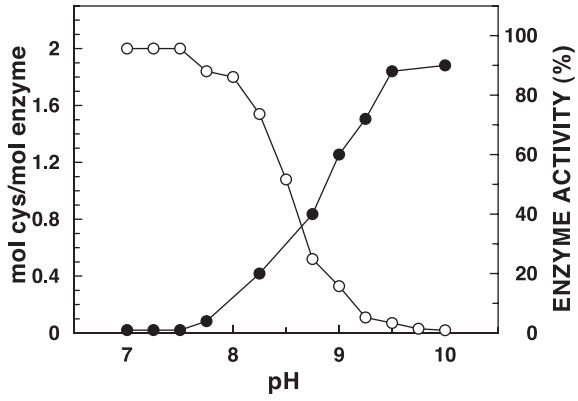

Fig. 4. Binding of [^{35}S]cystine to ASADH as a function of pH. The protein (1.4 μM) was incubated with 3 μM [^{35}S]cystine for 10 min at different pH values. The amount of [^{35}S]cysteine incorporated was determined after TCA precipitation of the protein. Aliquots were taken for determination of the remaining activity of the protein after removal of the unreacted cystine by ultrafiltration.

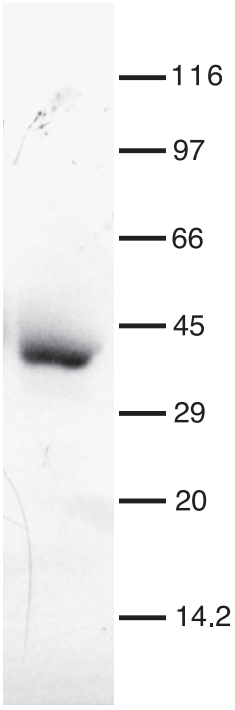

Fig. 5. Autoradiography of a sample of ASADH labeled with [^{35}S]cystine. Molecular weights of protein markers are given as a reference. Five micrograms of pure ASADH was treated with excess of [^{35}S]cystine at pH 7.5 for 20 min. The protein was precipitated with TCA and subjected to SDS-PAGE as described in Materials and methods.

nase. They also found that an acidic group with an apparent pK_a of 8.5 was involved in binding and concluded that such enzyme residue was a cysteine.

We have also examined the residual enzyme activity after cystine treatment at the same pH values used for the binding experiment. The experiment was performed in the same conditions as described above. Aliquots were taken and ultrafiltered in order to remove the unreacted cystine. The enzyme was then assayed using the standard assay conditions. At neutral pH values, the enzyme did not display activity, as explained above. When the pH increased, the enzyme displayed increasing activity. The activity displayed at the maximal pH value tested (pH 10) was around 90% of the initial enzyme activity (Fig. 4).

The reaction products were also investigated at pH values of 8.5 and 10, following the protocol described above. At pH 8.5, the amount of free [^{35}S]cysteine detected after TLC was around 50% of the amount detected at pH 7.5. No reaction products other than cysteine were detected. At pH 10, we were not able to detect free [^{35}S]cysteine, and all the initial [^{35}S]cystine remained unreacted. Though the reactivity of thiol groups is usually increased at basic pH values, in this case the expected effect is the opposite. We think that the decrease in the binding at basic pH could be due to modifications in the tertiary structure of the protein in the environment of the active center, which make this center less accessible to the interaction with the cystine.

3.5. Detection of [^{35}S]cystine-labeled ASADH in polyacrylamide gels

As a further relevant evidence on the stability of the complex formed during the reaction between [^{35}S]cystine and ASADH, a sample of the [^{35}S]cystine-treated enzyme was subjected to polyacrylamide gel electrophoresis in the presence of SDS. The enzyme samples were processed in the absence of thiol compounds (β-mercaptoethanol, DTT) in the sample buffer in order to avoid hydrolysis of the possible disulfide bridges formed between the amino acid and the enzyme. After electrophoresis, the gel was cut into two symmetrical halves. One half was vacuum-dried and exposed using a Hyperfilm-MP (Amersham) and the other half was stained using Coomassie blue. Fig. 5 shows that the radioactively labeled protein (ASADH used in this work was electrophoretically pure) migrated a distance corresponding to the molecular weight of the enzyme subunit (38,000 Da), thus corroborating that binding of radioactive cystine was to the ASADH protein and not to other polypeptides that could be present in the enzyme preparation as contaminant.

3.6. Protease digestion and peptide sequencing of the [^{35}S]-labeled ASADH

We finally tried to identify the amino acid residue from the ASADH enzyme which is modified by the reaction with the cystine. The [^{35}S]-labeled ASADH was digested in small-scale reactions using several proteases (trypsin, pepsin and staphylococcal V8 protease) and the size of the peptides

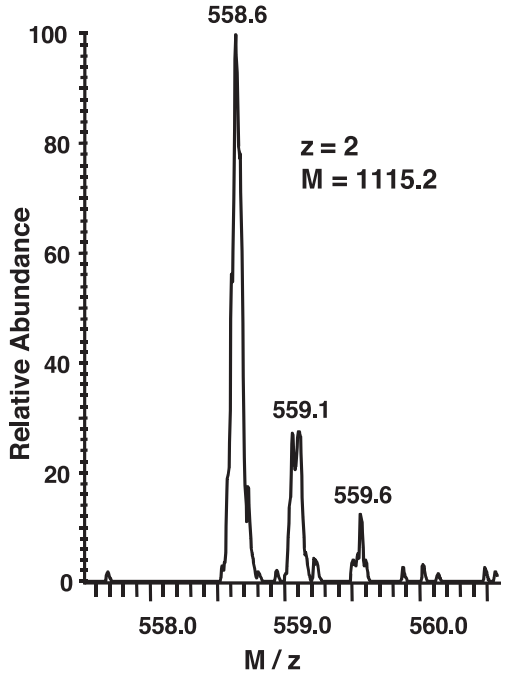

Fig. 6. Mass spectrometry analysis of the peptide from the pepsin digestion of the [^{35}S]-labeled ASADH.

generated was analyzed by SDS-PAGE. From this analysis, we chose pepsin for a large-scale digestion, according to the protocol described in Materials and methods. The peptides generated from the pepsin digestion were separated by HPLC and their radioactivity was monitored. A main peptide containing around 95% of the radioactivity bound to the enzyme was isolated and sequenced by the classical Edman degradation method. The sequence obtained by this method was FVGGNXTVSL.

During a study about affinity labeling of ASADH with L-2-amino-4-oxo-5-chloropentanoic acid, Savige and Fontana [10] found that this compound was bound to the enzyme. After digestion of the modified enzyme, they isolated a peptide containing a similar sequence to that mentioned above. However, they failed to identify the amino acid residue modified by the L-2-amino-4-oxo-5-chloropentanoic acid, and suggested that this residue was a histidine, based on the pH dependence of the inactivation reaction.

In order to characterize the unidentified residue, we proceeded to a mass spectrometry study of the peptide. The molecular ion corresponding to the peptide showed a mass/charge ratio of 558.6 (see Fig. 6). This value corresponds to a peptide with an estimated mass of 1115.2, which exactly matches the mass calculated for the peptide mentioned above containing cystine as the unidentified residue. Further fragmentation analysis of the peptide confirmed the sequence previously found by the Edman procedure and allowed unequivocal identification of the unknown residue as cystine. The final sequence, FVGGN$(Cys)_2$TVSL, exactly corresponds to the region containing the amino acids 130 to 139 in the sequence of the ASADH from *E.coli*. The amino acid in position 135, a cysteine, has been previously described to be essential for the enzyme activity [8]. Thus, we can conclude that the cystine inhibited the enzyme activity by binding to the essential ^{135}Cys of the enzyme.

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