Multiple Forms of Rat Liver Cysteinesulfinate Decarboxylase*

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Cysteinesulfinate decarboxylase, purified from male rat livers and homogeneous by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, is resolved into five distinct enzyme species (isoforms) by gel isoelectric focusing. Since the isoforms are present in fresh liver homogenates and do not arise by proteolysis, the enzyme is apparently heterogeneous in vivo. Although female rat livers contain only 5% of the cysteinesulfinate decarboxylase activity of male livers, immunological and enzymatic studies indicate that the distribution of isoforms is similar in both sexes. Rat brain and kidney also contain multiple isoforms which are cross-reactive with polyclonal antibodies prepared to the liver enzyme. The enzyme exhibits a protomer Mr, of 53,000, and the native enzyme is shown by cross-linking studies to be dimeric. Purified enzyme contains no carbohydrate or phosphate and does not bind excess pyridoxal 5'-phosphate.

Two pools of enzyme activity are resolved preparatively by chromatofocusing chromatography and have been examined with respect to substrate and inhibitor specificity. Both pools are most active toward L-cysteinesulfinate and L-cysteinesulfonate. Aspartate, homocysteinesulfinate, homocysteinesulfonate, 2-amino-3-phosphonopropionate, and glutamate are decarboxylated at rates less than 1% of that observed with L-cysteinesulfinate; D-cysteinesulfinate is not decarboxylated but is an effective inhibitor. The enzyme isoforms cannot be distinguished on the basis of substrate affinity or specificity. The enzyme is irreversibly inactivated by the mechanism-based inhibitors β-methylene-DL-aspartate and β-ethylidene-DL-aspartate. β-Ethylideneaspartate, in contrast to the β-methylene derivative, does not inhibit aspartate aminotransferase, an enzyme also important in cysteinesulfinate metabolism. β-Ethylidene aspartate or related β-ethylidene compounds may be useful in selectively altering cysteinesulfinate metabolism in vivo.

In mammals cysteine is subject to a variety of degradative reactions which together maintain the low in vivo concentration of the amino acid (1–4). In most species the formation of cysteinesulfinate, catalyzed by cysteine dioxygenase, accounts for a substantial fraction of cysteine metabolism and initiates the major pathway of taurine biosynthesis. Further metabolism of cysteinesulfinate occurs both by transamination, catalyzed by aspartate aminotransferase, and by decarboxylation, catalyzed by cysteinesulfinate decarboxylase. Only the latter reaction leads ultimately to taurine; transamination of cysteinesulfinate yields pyruvate and sulfite. The activity of cysteinesulfinate decarboxylase and thus presumably the partitioning of cysteinesulfinate between decarboxylation and transamination is subject to wide species, sex, hormonal, and developmental variations (3–12). Whereas humans (10, 13) and adult cats (5, 13) have, for example, little capacity for cysteinesulfinate decarboxylation, adult male mice decarboxylate about 80% of exogenously supplied cysteinesulfinate and oxidize >90% of the resulting hypotaurine to taurine (14). Elucidation of factors controlling the partitioning of cysteinesulfinate and the development of compounds which can predictably alter the decarboxylation/transamination ratio in vivo are of considerable interest since an adequate supply of taurine is required for normal bile acid formation, plasma membrane stabilization, neurotransmission, and, in the cat, vision (15–18).

In previous studies from this laboratory (14), rat liver cysteinesulfinate decarboxylase was purified to near homogeneity and was shown to consist of at least two distinct protein species which could be resolved by chromatofocusing. The enzyme was shown to be inhibited in vitro and in vivo by β-methylene DL-aspartate (14, 19), a mechanism-based inhibitor which also inactivates aspartate aminotransferase (20). In the present studies, rat liver cysteinesulfinate decarboxylase has been purified to homogeneity as judged by SDS-PAGE. The final specific activity is 4–20-fold higher than reported previously for apparently homogeneous enzyme (14, 21, 22). The purified enzyme is shown to consist of multiple protein species, and the distribution of these species in rat kidney and brain has been examined using polyclonal antibodies. Cross-linking experiments are reported which resolve previous ambiguities regarding the molecular weight and subunit composition of the native enzyme. We have also examined the substrate specificity and kinetics of the enzyme and have characterized its inactivation by β-ethylideneaspartate, an analog of β-methyleneaspartate that exhibits greater specificity of inhibition.

1 The abbreviations used are: SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high performance liquid chromatography; L, liter; BSA, bovine serum albumin; MOPS, 4-morpholino propane sulfonic acid.

2 Comparison of the specific activity of the crude homogenate reported in Ref. 22 with that observed in the present studies suggests that the units definition in Ref. 22 is mis-stated; the final specific activity observed in those studies was probably 4.2 μmol·min⁻¹·mg⁻¹ rather than 0.19 μmol·min⁻¹·mg⁻¹ as reported by the authors.

3 Preliminary reports of some of the present results have appeared (23–25).

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**TABLE I**

| Step                        | Volume | Total protein | Activity* | Yield |
|-----------------------------|--------|---------------|-----------|-------|
| 1. Crude homogenate         | 6,000  | 106,000       | 1,840     | 100%  |
| 2. 7,000 × g supernatant    | 5,340  | 54,400        | 1,770     | 97%   |
| 3. pH 5.6 supernatant       | 5,100  | 24,700        | 1,610     | 88%   |
| 4. 50% ammonium sulfate pellet | 420   | 4,200         | 1,730     | 94%   |
| 5. Phenyl-Sepharose pool    | 1,580  | 506           | 985       | 57%   |
| 6. Chromatofocusing pool    |        |               |           |       |
| Major                       | 82     | 154           | 637       | 35%   |
| Minor                       | 62     | 25            | 63        | 3.4%  |
| 7. Ultrogel AcA 44 pool     |        |               |           |       |
| Major                       | 4      | 15            | 230%      | 13%   |
| Minor                       | 3.7    | 8             | 27        | 1.5   |

*1 unit of activity forms 1 μmol of product per min.

*The two fractions of highest specific activity were pooled. Three additional fractions contained 212 units (specific activity = 13.3) for a total of 442 units (24% yield).

**RESULTS**

**Purification of Cysteinesulfinate Decarboxylase**—Cysteinesulfinate decarboxylase was purified 900-fold from male rat liver to a final specific activity of 15.2 units/mg (range in 9 preparations: 11–16 units/mg); the overall yield was 25.5% (Table I). The first four steps are similar to those described previously (14) but incorporate several modifications, including a simplified preparation of cytoplasmic supernatant, an earlier neutralization of the pH 5.6 supernatant, and the collection of enzyme over a wider ammonium sulfate saturation range. The chromatographic procedures are significantly improved by elimination of two gel filtration procedures and a low-yield ion-exchange chromatography; chromatography on phenyl-Sepharose is now employed and gives a 4.9-fold purification with a moderately good yield. As noted earlier (14), a major and minor activity peak are separated by chromatofocusing chromatography. In the present studies, the minor peak constitutes 8–10% of the total activity and elutes at pH 5.3; the major activity peak elutes at pH 5.6 (Fig. 1 (Miniprint)).

Cysteinesulfinate decarboxylase purified from the major chromatofocusing activity pool elutes from a calibrated Ultrogel AcA 44 column with an apparent Mr of 60,000 (see “Experimental Procedures” (Miniprint)).

To determine unambiguously whether the native enzyme is monomeric or dimeric, cysteinesulfinate decarboxylase was treated with dimethyl suberimidate to covalently cross-link the subunits of any polymeric species present. Aliquots were removed from the resulting solution at intervals, and the protein species present were fractionated on the basis of size by SDS-PAGE (Fig. 3). As shown, the untreated enzyme exhibits only protoemns of Mr 53,000 (lane 1), but within 15 min of adding dimethyl suberimidate significant formation of a dimeric species of apparent Mr 98,000 is evident (lane 2). A cross-linking is allowed to continue, two additional bands with apparent Mr, values of about 78,000 and 86,000 also appear, and the band corresponding to uncross-linked Mr 53,000 subunits continues to decrease and almost disappears (lanes 3–6). Since the relative intensity of the Mr 98,000 species also begins to decrease after 30 min (lanes 4–6), it is probable that the Mr, 86,000 and Mr 78,000 bands represent dimeric species present as “knots” in which multiple interchain and intrachain cross-links prevent complete unfolding in the presence of SDS. Similarly, the faint band with an apparent Mr of 35,000, seen most intensely in lanes 3 and 4, is likely to represent monomer with one or more intrachain cross-links. Knotted monomers and polymers exhibiting anomalously high migration rates have been identified in other cross-linking studies (27).

**Molecular Weight and Quaternary Structure of Native Cysteinesulfinate Decarboxylase**—The native molecular weight of cysteinesulfinate decarboxylase has been previously estimated by gel filtration on both Sephadex G-100 (Mr = 66,000 (21) or 65,000–70,000 (26)) and Bio-Gel P-150 (Mr = 78,000–85,000 (26)). In contrast, subsequent studies using analytical ultracentrifugation or nondenaturing PAGE suggested that the native enzyme has an Mr of 96,000 or 105,000 ± 4,000, respectively, and it was concluded that the native enzyme is a dimer of Mr 50,000–55,000 subunits (22). In the present studies, we find that cysteinesulfinate decarboxylase from both the major and minor chromatofocusing activity pools exhibits a single protein band on SDS-PAGE with Coomassie Blue or silver staining. In identical unstained gels enzyme migrates identically to enzyme purified from the major chromatofocusing activity pool.

The subunit molecular weight of cysteinesulfinate decarboxylase was estimated by gel filtration on a calibrated Ultrogel AcA 44 column with an apparent Mr of 60,000 (see “Experimental Procedures” (Miniprint)).
major chromatofocusing activity peak, homogeneous by SDS-PAGE, was separated into several major and minor protein bands by electrofocusing over the pH range 4–6.5 on polyacrylamide gels (Fig. 4A). The one minor and three major protein bands have pI values of 5.6, 5.3, 5.1, and 5.0; together they constitute >90% of the total protein as judged by either Coomassie Blue or silver staining. Gel lanes run in parallel and not stained were sliced into segments, and the gel segments were assayed for cysteinesulfinate decarboxylase. All of the above-mentioned protein bands contain enzyme activity in amounts approximately proportional to their protein-staining intensity (Fig. 4A); the resolution of the segmenting procedure is insufficient to determine whether the very minor protein bands also contain enzyme activity. In similar experiments using enzyme purified from the minor chromatofocusing activity peak only one protein (pI 4.9) of the several separated by gel electrofocusing is found to contain significant cysteinesulfinate decarboxylase activity (Fig. 4B). The protein band corresponding to this activity is not seen on gels loaded with enzyme isolated from the major chromatofocusing activity peak (i.e. in Fig. 4A, activity at pH values <5.0 is apparently due to spreading of the pI 5.0 and 5.1 species and does not reflect the presence of the pI 4.9 species).

Livers from female rats contain much less cysteinesulfinate decarboxylase activity than do male rat livers (5, 7, 28). To examine its possible heterogeneity, the enzyme was isolated from female rat livers using the procedures outlined in Table 1. Major and minor activity peaks were separated by chromatofocusing, and enzyme isolated from the major peak was found to be about 75% pure by SDS-PAGE with an apparent $M_r$ of 53,000. The final specific activity was 3.8. Examination of the purified female rat liver enzyme by gel electrofocusing indicates that the distribution of enzyme isoforms is qualitatively and quantitatively very similar to that seen with enzyme purified from male liver (Fig. 4C). It should be noted that, although the specific activity of the purified female liver enzyme is low, the initial homogenate from female livers contained only 5% of the activity found in male livers. Since cysteinesulfinate decarboxylase is unstable in dilute solution, the lower final specific activity of the female rat enzyme is probably attributable to proportionately greater inactivation during purification.

Since the occurrence of multiple, closely related enzyme species may be due to post-translational alterations of a single gene product, male rat liver enzyme isolated from the major chromatofocusing activity pool has been examined for evidence of either in vivo or artificial protein modifications. Basic fuchsin, a reagent specific for glycoproteins (29), does not stain the single, unfractionated band observed on SDS-PAGE. Similarly, treatment of the enzyme with neuraminidase does not alter the gel electrofocusing pattern. The several protein species do not appear to represent differing phosphorylation states since treatment with alkaline phosphatase (30) does not alter the relative intensity of the several protein bands, and no phosphoserine or phosphothreonine is detected following acid hydrolysis of the purified enzyme. Although pyridoxal 5'-phosphate, a cofactor sometimes included in the enzyme buffers (Refs. 14 and 23, and "Experimental Procedures"), is reportedly able to bind artifically to multiple lysine residues on the surface of some proteins, the same gel electrofocusing patterns are observed in enzyme isolated in the presence or absence of pyridoxal 5'-phosphate. No evidence for excess co-factor is found by direct assay (see below). The several protein species also do not appear to arise by proteolysis. For example, enzyme partially purified from crude liver homogenates that were allowed to stand 2 h at 25 °C was compared with enzyme from liver homogenates rapidly prepared and partially purified in the presence of a spectrum of protease inhibitors; both enzyme preparations were fractionated similarly by isoelectric focusing as judged by assay of gel segments. Supernatants obtained by brief high-speed centrifugation of fresh liver homogenates have been submitted directly to gel electrofocusing and are found to show activity profiles similar to those shown in Fig. 4A. Consistent with the absence of proteolysis, no free N-terminal amino acids were found in an attempt to sequence the mixed pI 5.0–5.6 enzyme pool purified from the major chromatofocusing activity peak. Details of these studies are given under "Experimental Procedures."

The possible interconvertibility of the several cysteinesulfinate decarboxylase isoforms has been examined by isolating gel segments containing specific protein bands and then re-evaluating the composition of the proteins in the isolated segment by repeat gel electrofocusing. Under all conditions tested, the isolated pI 5.6 and the pI 5.3 species focus as single bands and give no evidence of being converted to any other species. The pI 5.0 and 5.1 species are separated by only 1–2 mm on the original gels and are thus cross-contaminated when gel segments are separated. Although a variable amount of pI 5.1 species is seen when the pI 5.0 species is rerun, there is no detectable formation of the pI 5.3 or pI 5.6 species. In contrast, electrofocusing gels loaded with isolated pI 5.1 species have repeatedly shown small amounts (2–10% of the protein loaded) of the pI 5.3 species in addition to a predominant band corresponding to the pI 5.1 species (variable amounts of protein in the pI 5.0 band are also seen but are attributed to contamination of the original isolate). As originally isolated, enzyme in the minor chromatofocusing activity pool contains only small amounts of the pI 5.0–5.6 species (Fig. 4B); over a period of weeks, the amount of the higher pI forms does not increase relative to the main pI 4.9 form.

**Tissue Distribution of the Multiple Forms of Cysteinesulfinate Decarboxylase**—Although liver contains most rat cysteinesulfinate decarboxylase, kidney and brain also contain significant enzyme activity. Supernatants obtained following high-speed centrifugation of liver, kidney, and brain were compared after fractionation by gel electrofocusing. When gel segments corresponding to the various protein bands were
assayed for activity, all tissues were found to exhibit distinct peaks of activity focusing at pH 5.3–5.2 and at pH 5.0–4.9. There is also activity in the region corresponding to the pl 5.6 species, but the enzyme activity centered at pH 5.2–5.3 tails through this region. Interpretation of the activity profile in this region is ambiguous. Glutamate decarboxylase activity was detected on the brain gels at pH values greater than 6.1 and did not overlap the cysteinesulfinate decarboxylase activity.

To facilitate the detection of cysteinesulfinate decarboxylase in homogenates, enzyme purified to homogeneity from the major chromatofocusing activity pool of male rat livers was used to produce polyclonal antibodies in rabbits. The IgG fraction obtained from serum by ammonium sulfate fractionation was purified by chromatography on an affinity resin containing liver cysteinesulfinate decarboxylase. By several criteria, the antibody preparation was found to react strongly and specifically with the enzyme. As shown in Fig. 5 (Miniprint), Western blot analysis of SDS-PAGE gels indicates that the purified antibody reacts with only a single species ($M_r = 53,000$) in crude male rat liver homogenates; preimmune serum is nonreactive. Similar studies with kidney and brain homogenates give similar results. Cysteinesulfinate decarboxylase activity is completely removed from crude liver homogenates after reaction with the affinity-purified antibody preparation and removal of antigen-antibody complexes with Protein A-agarose gel. Used similarly, preimmune serum has no effect. Even without removing the antigen-antibody complexes, the decarboxylase activity of purified enzyme is inhibited in a concentration-dependent manner by the IgG fraction; inhibition was $>95\%$ at the highest antibody to enzyme ratios tested.

Centrifuged homogenates from female rat liver and male rat liver, kidney, and brain were fractionated by gel isoelectric focusing to separate the multiple enzyme forms; the separated proteins were blotted onto nitrocellulose. In all tissues examined, the purified antibody reacted with protein bands corresponding to all of the enzyme forms present in cysteinesulfinate decarboxylase purified from male rat liver. In a similar experiment, 10 individual liver homogenates were prepared from single rats, and the proteins in each were fractionated by gel electrophoresing. For each rat, polyclonal
antibodies visualized all of the several forms of cysteinesulfinate decarboxylase; an individual rat thus produces all isoforms of the enzyme.

Since the distinction between brain glutamate decarboxylase and cysteinesulfinate decarboxylase has remained controversial, rat brain homogenates were purified through step 5 (phenyl-Sepharose chromatography) of the protocol developed for rat liver. The partially purified protein was examined by gel isoelectric focusing and found to contain an active enzyme species focusing at pl 5.6 and multiple species focusing in the region between pH 5.4 and 5.3 (Fig. 4D). Since glutamate decarboxylase is known to decarboxylate cysteinesulfinate as an alternative substrate (30, 31), cysteinesulfinate decarboxylase was routinely assayed in both the presence and absence of a large excess of unlabeled glutamate. In crude brain homogenates, glutamate inhibited the decarboxylation of 10 mM [35S]cysteinesulfinate about 50%, a finding which suggests that glutamate decarboxylase accounts for about one-half of the activity detected at this high substrate concentration. Glutamate was without effect on the partially purified enzyme (step 5). In other experiments 50% of the total cysteinesulfinate decarboxylase activity, corresponding to about 100% of the glutamate-insensitive activity, was removed from crude brain homogenates by reaction with affinity-purified antibody followed by Protein A-agarose.

Cysteinesulfinate decarboxylase was purified through step 5 from male rat kidney and examined by gel electrofocusing. Protein staining disclosed several dozen bands, but assay of slices from a gel lane run in parallel localized enzymatic activity to the same general regions seen with the liver enzyme. Thus, rat kidney contains cysteinesulfinate decarboxylase species that focus at pH values of 5.6 (about 1% of the total activity), 5.3-5.2, (about lo%), and 5.1-4.9 (about 90%).

Cysteinesulfinate decarboxylase activity toward the two substrates is thus not altered by enzyme purification. In other experiments inhibition by thiol-binding agents (22), and activation by assay in nitrogen-purged reaction mixtures (23) are also examined as potential inhibitors of cysteinesulfinate decarboxylase species purified from the major and minor chromatofocusing activity pools cannot be distinguished on the basis of susceptibility to any of the several inhibitors tested.

Inhibition of Cysteinesulfinate Decarboxylase by P-Alkylidene-DL-aspartate—Previous studies indicated that both cysteinesulfinate and cysteinesulfonate are moderately good inhibitors; at concentrations of 0.5 mM, each inhibits the decarboxylation of 0.25 mM [35S]cysteinesulfinate by >14%. α-Methyl-DL-cysteinesulfinate shows somewhat less inhibitory activity; previous studies indicated that α-methyl-DL-cysteinesulfonate is not an effective inhibitor (14). Cysteinesulfinate decarboxylase species purified from the major and minor chromatofocusing activity pools is not distinguished by gel electrofocusing.

Substrate Specificity of Cysteinesulfinate Decarboxylase Isozymes—As shown in Table II, the pooled major chromatofocusing isozymes (pl 5.0, 5.1, 5.3, and 5.6) and the minor chromatofocusing isozyme (pl 4.9) have similar and quite restricted substrate specificities with respect to cysteinesulfinate analogs. Cysteinesulfinate and cysteinesulfonate are effectively decarboxylated, aspartate, 2-amino-5-phosphono-propionate, glutamate, homocysteinesulfinate and homocysteinesulfonate are also decarboxylated but at much lower rates. The various substrate analogs and related compounds were also examined as potential inhibitors of cysteinesulfinate decarboxylase (Table III). d-Cysteinesulfinate, a non-substrate, and l-cysteinesulfonate, a good alternative substrate, are moderately good inhibitors; at concentrations of 5 mM, each inhibits the decarboxylation of 0.25 mM l-[35S]cysteinesulfinate by >14%. α-Methyl-DL-cysteinesulfinate shows somewhat less inhibitory activity; previous studies indicated that α-methyl-DL-cysteinesulfonate is not an effective inhibitor (14). Cysteinesulfinate decarboxylase species purified from the major and minor chromatofocusing activity pools is not distinguished by gel electrofocusing.
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Substrate specificity of enzyme in the major and minor chromatofocusing pools

Cysteinesulfinate decarboxylase, isolated from the major (pI 5.0-5.6) or minor (pI 4.9) chromatofocusing pool, was added to reaction mixtures containing the compounds indicated, and the formation of the corresponding decarboxylation product was determined at intervals by high performance liquid chromatography as described under "Experimental Procedures." In all cases the formation of product was linear with time.

| Substrate analog | Activity | pI 5.0-5.6 species | pI 4.9 species |
|------------------|----------|--------------------|--------------|
|                  | μmol·min⁻¹·mg⁻¹ | % | μmol·min⁻¹·mg⁻¹ | % |
| 1. L-Cysteinesulfinate | 14.5 ± 0.7 | 100 | 6.52 ± 0.23 | 100 |
| 2. D-Cysteinesulfinate | <0.0005 | ND | <0.0005 | ND |
| 3. L-Cysteinesulfinate | 1.18 ± 0.12 | 8.0 | 0.08 ± 0.11 | 10.6 |
| 4. L-Homocysteinesulfinate | 0.0092 ± 0.0002 | 0.02 | 0.0009 ± 0.0002 | 0.013 |
| 5. L-Homocysteinesulfinate | 0.005 ± 0.004 | 0.03 | 0.008 ± 0.008 | 0.1 |
| 6. L-Aspartate | 0.08 ± 0.03 | 0.6 | 0.03 ± 0.01 | 0.5 |
| 7. L-Glutamate | 0.002 ± 0.0002 | 0.01 | 0.001 ± 0.0001 | 0.02 |
| 8. DL-2-Amino-3-phosphonopropionate | 0.003 ± 0.0001 | 0.02 | 0.00733 ± 0.00002 | 0.112 |
| 9. L-Penacilaminosulfonate | <0.0005 | ND | <0.0005 | ND |
| 10. α-Methyl-DL-cysteinesulfonate | <0.0005 | ND | <0.0005 | ND |

* ND, none detected.

Inhibition of cysteinesulfinate decarboxylase by substrate analogs

Cysteinesulfinate decarboxylase, isolated from the major (pI 5.0-5.6) or the minor (pI 4.9) chromatofocusing pool, was reacted with 0.25 mM L-[³⁵S]cysteinesulfonate in the presence or absence of the substrate analogs indicated. Analogs were present at the concentrations shown; the complete protocol is given under "Experimental Procedures." Formation of [³⁵S]hypotaurine was evaluated at 7.5 and 15 min and the extent of inhibition was determined. In all cases, the time course of the reaction remained linear indicating that irreversible inhibition did not occur. The results presented are averages of 3-14 independent determinations and are expressed as percent inhibition in comparison to a reaction mixture containing only substrate. The comparison of the pI 5.0-5.6 enzyme to the pI 4.9 enzyme for each of the compounds tested indicated no significant differences (p > 0.1).

| Substrate analog (mM) | Percent inhibition | pI 5.0-5.6 enzyme | pI 4.9 enzyme |
|-----------------------|--------------------|-------------------|--------------|
| 1. D-Cysteinesulfinate (5) | 86.0 ± 2.4 | 86.6 ± 5.6 |
| 2. L-Cysteinesulfinate (5) | 74.0 ± 5.5 | 77.1 ± 5.2 |
| 3. L-Homocysteinesulfinate (5) | 0.2 ± 0.54 | 0.3 ± 2.3 |
| 4. L-Homocysteinesulfinate (5) | 10.9 ± 4.7 | 10.8 ± 6.4 |
| 5. β-DL-Homocysteinesulfinate (10) | 16.9 ± 3.9 | 20.1 ± 5.3 |
| 6. α-Methyl-DL-cysteinesulfinate (10) | 30.0 ± 8.5 | 30.7 ± 6.5 |
| 7. L-Aspartate (5) | 16.8 ± 4.2 | 20.2 ± 4.0 |
| 8. L-Glutamate (5) | -0.8 ± 3.9 | -3.7 ± 4.8 |
| 9. DL-2-Amino-3-phosphonopropionate (10) | 16.0 ± 4.7 | 14.5 ± 2.5 |

DISCUSSION

The occurrence of multiple forms of cysteinesulfinate decarboxylase and the relationship of those enzymes to cysteinesulfonate decarboxylase and glutamate decarboxylase have been considered in several previous studies. An early suggestion by Awapara and Wingo (35), that cysteinesulfinate and cysteinesulfonate are decarboxylated by the same enzyme, has been questioned (21, 36) but is sustained by both early (33) and recent investigations (22) including those reported here. There is much less agreement regarding the number of "isozymes" of cysteinesulfinate decarboxylase present in liver and brain. Previous studies in which the rat liver enzyme was purified to near or apparent homogeneity have identified a single protein species (22), two species separable by chromatofocusing (14), or three species separable by Sephadex G-100 and DEAE-cellulose chromatography (21). Studies of brain cysteinesulfinate decarboxylase are complicated by the presence of large amounts of glutamate decarboxylase, an enzyme with activity toward both cysteinesulfinate and cysteinesulfonate (31, 32). Recent investigations indicate, however, that brain contains a distinct decarboxylase that is specific for cysteinesulfinate and cysteinesulfonate (37-39); this enzyme has a much higher affinity for cysteinesulfinate (Km = 0.18 mM) than does glutamate decarboxylase (Km = 5.2 mM) (37). Two forms of brain cysteinesulfinate decarboxylase have been resolved by hydroxyapatite (38) or DEAE-cellulose (37) chromatography; glutamate decarboxylase was identified as a third and distinct cysteinesulfinate decarboxylating activity in both studies (37, 38).

Results reported here indicate that rat liver cysteinesulfitinate decarboxylase can be separated into five isoforms by isoelectric focusing on acrylamide gels; the observed isoelectric points range from 5.6 to 4.9 with most of the activity initially present as the pI 5.3 and 5.1 species. As reported here and previously (14), the pI 4.9 species can be resolved from the other species by chromatofocusing; the other species have to date been resolved only by gel electrophoresing. In earlier studies, Guion-Rain et al. (22) found rat liver cysteinesulfinate decarboxylase to band as a single species with a pI of 5.1 ± 0.1 during cellulose acetate electrophoresis; this observation is reasonably consistent with the present findings if it is assumed that the pI 5.3-5.0 species, representing most of the activity, were not resolved and that the other species were too minor to detect. Although Lin et al. (21) report the resolution of three cysteinesulfinate decarboxylase species by DEAE-cellulose and Sephadex G-100 chromatography, we have been
**Panel A.**

**Fig. 6. Inhibition of cysteinesulfinate decarboxylase by β-ethylidene-DL-aspartate.** Enzyme purified from the major chromatofocusing activity pool (0.76 unit) was first activated by incubation with dithiothreitol, and small aliquots were then added to reaction mixtures (final volume, 200 μl) containing 200 mM K+ phosphate buffer, pH 6.8, 0.1 mM pyridoxal 5'-phosphate, and 0–40 mM β-ethylidene-DL-aspartate, as indicated. At intervals, 25-μl portions of those mixtures were assayed for residual enzyme activity. **Panel A** shows the loss of activity with time; lines 1, 2, 3, and 4 correspond to reaction mixtures containing 5, 10, 20, and 40 mM β-ethylidene-DL-aspartate, respectively (there was no loss of activity in the absence of inhibitor). **Panel B** is a reciprocal plot showing the relationship between inhibitor concentration and kact, the pseudo-first order rate constant for inactivation determined from the slopes of the lines in panel A.

**Panel B.**

The physical basis for the occurrence of multiple forms of cysteinesulfinate decarboxylase has been examined in several studies reported here. The finding that all of the enzyme species can be detected both immunologically and by activity in a rapidly prepared supernatant of crude liver homogenate strongly suggests that the several species occur in vivo. Since all isoforms have the same native and subunit M, and since the relative proportions of the several species are unchanged in homogenates "aged" in the presence of endogenous proteases, the various isoforms are unlikely to be generated by proteolysis. Consistent with this view, we have been unable to identify any N-terminal amino acids in the purified but unresolved major chromatofocusing enzyme species; the pI 5.0–5.6 species thus appear to all have an intact and blocked N terminus. By direct examination we have shown that the unfractionated cysteinesulfinate decarboxylase species do not contain carbohydrate, phosphate, or excess pyridoxal 5'-phosphate; the occurrence of multiple enzyme forms is thus not accounted for by differences in the extent of those modifications.

Relatively few additional explanations for the occurrence of multiple enzyme forms are known; of these, genetic polymorphism as well as differences based on pseudo-stable conformational states or on partial deamidation of glutamine and asparagine residues warrant particular consideration (40). To date, several attempts to distinguish among these possibilities have been unsuccessful. Thus, individual male rat livers do not contain a subset of the enzyme forms found in pooled liver samples, and the lower overall activity in female rat liver is not attributable to the expression of only a single isozyme. Opposite results in either study would have strongly suggested the existence of multiple alleles for cysteinesulfinate decarboxylase; the actual findings constrain genetic explanations but do not eliminate them. Preliminary peptide mapping studies offer no support for the possibility of genetic polymorphism or for the possibility that the several enzyme species are formed post-translationally by partial deamidation. For example, comparison of the peptides formed from the purified pI 5.0–5.6 isoforms with peptides formed from the pooled pI 4.9 species show no marked differences; in particular, the pooled pI 5.0–5.6 isoforms did not form a much larger number of peptides as would have been expected if variable deamidation accounted for the multiplicity of enzyme forms (data not shown). Alternatively, if the multiplicity of enzyme species were due to conformational isomerism, the peptide maps should not differ, but interconversion of the various species could be possible under some conditions (41–43). Although we find no evidence for the general interconvertibility of the various cysteinesulfinate decarboxylase species, there is limited but reproducible formation of the pI 5.3 species when the pI 5.1 species is eluted from isolectric focusing gels and rerun. This single instance of interconvertibility is suggestive of conformational isomerism but cannot alone sustain that explanation for all of the enzyme species. Additional sequencing and peptide mapping studies may establish the physical basis of enzyme heterogeneity, but such studies require the prior development of methods able to resolve significant amounts of individual isomers.

The relationship between liver cysteinesulfinate decarboxylase and the enzyme present in other tissues has been examined by both direct enzymatic assay and immunological studies. In the rat, as in most species examined (5, 6, 10, 11, 13), the enzyme is most active in liver but is present in substantial amounts in kidney and brain. In the present studies, enzyme was readily detectable by both activity and immunological assay in rat lung, spleen, and stomach; trace amounts of activity were detected in skeletal muscle and heart (data not shown). All five enzyme species present in liver are also present in rat brain and kidney. Although we also detect glutamate decarboxylase in rat brain, this protein is resolved by isolectric focusing from all cysteinesulfinate decarboxylase species.

As noted, cysteinesulfinate and cysteinesulfonate are both substrates of most previously reported preparations of cysteinesulfinate decarboxylase. As reported here, the ratio of cysteinesulfinate to cysteinesulfonate activity is, within experimental error, constant in the major enzyme species resolved by isolectric focusing (Table IV). Reports by other investigators (21) of enzyme preparations showing activity only toward cysteinesulfinate cannot, therefore, be attributed to the selective and inadvertent purification of any of the several species identified here; the explanation of the previous...
findings remains obscure. Examination of enzyme activity toward a variety of additional cysteinesulfinate analogs (Table II) establishes that the enzyme is very specific. Activity is almost completely lost if the side chain is extended by one methylene group or if the anionic -SO₂ or -SO₃ groups are replaced by carboxylates or phosphonates. Inhibition studies (Table III) indicate that the poor activity of most analogs can be attributed at least in part to their poor binding; most analogs do not compete effectively with cysteinesulfinate for binding and are thus also poor inhibitors. The exception is β-cysteinesulfinate, which binds well but is not decarboxylated. It is probable that correct and strong binding of the amino and sulfinate groups of β-cysteinesulfinate places the carboxylate outside of the normal active site, and its decarboxylation is not facilitated. The observation that β-ethylidene-DL-aspartate is an effective and relatively specific inhibitor of cysteinesulfinate, which binds well but is not decarboxylated.

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Additional references are found on p. 7263.
Cysteinesulfinate Decarboxylase

**Supplemental Material to Multiple Sources of Rat Liver Cysteinesulfinate Decarboxylase**

BY C. E. C. Miller & C. F. Unanue

**Materials and Synthetic Procedures**

Biochemical reactions and reagents for organic synthesis were obtained from Sigma and Aldrich Chemical Co. and were used as received, except where noted. Organophosphorus reagents were purchased from Fisons (U.K.), Merck, and J. T. Baker Chemical Co. Chloroform and tetrahydrofuran were obtained from Burdick and Jackson Laboratories. Deuterated solvents were purchased from Cambridge Isotope Laboratories. All other solvents were purchased from Mallinckrodt. 

**Methods**

**Enzymatic Assays**

The enzyme was purified as described previously. 

**Results**

1. The enzyme was isolated from rat liver... 

2. The enzyme was assayed by... 

3. The enzyme was characterized by... 

**Discussion**

The enzyme was found to be... 

**Conclusion**

The enzyme is characterized by... 

**Supplemental Data**

Additional data supporting the conclusions are provided in...
Cysteinesulfinate Decarboxylase

![Image showing the chromatography of cysteinesulfinate decarboxylase on a column.](image)

**Figure 3**: Gel electrophoresis of cysteinesulfinate decarboxylase.

The results of experiments 1 and 2 were summarized in Table IV.

**Table IV**: Cysteinesulfinate and Cysteine-Dependent Enzyme Activities of Individual Enzyme Forms of the Decarboxylase

| Enzyme Form | Activity (moles/min) x 10^5 | Activity Ratio |
|-------------|----------------------------|---------------|
| Control     | 1.7                        | 1.00          |
| Experiment 1| 1.5                        | 0.88          |
| Experiment 2| 1.4                        | 0.82          |

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