Mechanisms involved in the regulation of key enzymes of cysteine metabolism in rat liver in vivo

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Mechanisms involved in the regulation of key enzymes of cysteine metabolism in rat liver in vivo. Am. J. Physiol. 276 (Endocrinol. Metab. 39): E326–E335, 1999.—Little is known about mechanisms of regulation of cysteine dioxygenase (CDO), γ-glutamylcysteine synthetase (GCS), and cysteine-sulfinate decarboxylase (CSDC) in response to diet. Enzyme activity and Western and Northern or dot blot analyses were conducted on liver samples from rats fed a basal low-protein diet or diets with graded levels of protein or methionine for 2 wk. Higher levels of CDO activity and CDO protein but not of CDO mRNA were observed in liver of rats fed methionine or protein-supplemented diets, indicating that CDO activity is regulated by changes in enzyme concentration. Lower concentrations of the heavy or catalytic subunit of GCS (GCS-HS) mRNA and protein, as well as a lower activity state of GCS-HS in rats fed methionine- or protein-supplemented diets, indicated that dietary regulation of GCS occurs by both pretranslational and posttranslational mechanisms. Lower CSDC activity, CSDC protein concentration, and CSDC mRNA concentration were found in rats fed the highest level of protein, and regulation appeared to involve changes in mRNA concentration. Regulation of key enzymes of cysteine metabolism in response to diet determines the use of cysteine for synthesis of its essential metabolites.

γ-glutamylcysteine synthetase; cysteine dioxygenase; cysteine-sulfinate decarboxylase; rats

TWO AMINO ACIDS that are incorporated into proteins, methionine and cysteine, contain a sulfur atom in their side chains. Methionine is utilized in protein synthesis and, via its metabolites, serves as a methyl donor in transmethylation reactions and as an aminopropyl donor in the synthesis of polyamines. The metabolism of methionine sulfur occurs predominantly via the transmethylation-transsulfuration pathway and results in the formation of cysteine. Cysteine is also utilized in the synthesis of proteins and for the synthesis of other essential nonprotein compounds.

Metabolites of cysteine include glutathione (GSH), inorganic sulfur, and taurine, which are essential for a wide variety of critical functions in the body (Fig. 1). GSH is involved in maintenance of the cellular thiol-disulfide ratio, serves as a cosubstrate in various enzymatic reactions, including those catalyzed by GSH sulfur-transferases and peroxidases, and serves as a reservoir for cysteine. Reduced sulfur is required for the synthesis of certain macromolecules, such as iron-sulfur proteins. Sulfate (in the form of 3-phosphoadenosine 5′-phosphosulfate) is required for numerous sulfation reactions, including the sulfation of glycosaminoglycans and the formation of sulfate esters of drugs as a detoxification mechanism. The role of taurine in bile acid conjugation is well known, whereas the specific role of taurine in other processes has not been clearly elucidated.

The activities of key regulatory enzymes involved in cysteine metabolism, cysteine dioxygenase (CDO; EC 1.13.11.20), γ-glutamylcysteine synthetase (GCS; EC 6.3.2.2), and cysteine-sulfinate decarboxylase (CSDC; EC 4.1.1.29), have been observed to change in liver of rats fed different levels of dietary protein (2, 5, 6). These changes in enzyme activities have been shown to occur in response to protein and not to the accompanying changes in the levels of other dietary macronutrients (4). In addition, the activities of CDO and GCS have been shown to change in response to changes in dietary methionine and/or cyst(e)ine levels (3, 5, 6). CDO increased in a dose-dependent manner in response to increasing levels of dietary methionine or protein (5, 6). In contrast, GCS decreased in a dose-dependent manner in response to increasing levels of dietary protein (4) or of supplemental methionine (H. Delans and M. H. Stipanuk, unpublished observations). Changes in CDO and GCS activities in response to increasing levels of dietary protein appear to be reciprocally and coordinately regulated, and diet plays a role in determining the flux of cysteine between cysteine catabolism and GSH synthesis (4). CSDC activity also decreases in a dose-dependent manner in response to increasing dietary protein levels, but it does not consistently decrease in response to increased dietary methionine levels (2, 5, 6, 17, 18).

Although numerous studies have shown that these three enzymes of cysteine metabolism respond to changes in dietary protein and/or methionine, little is known about the possible mechanism(s) of regulation of CDO and GCS in response to diet. Yamaguchi et al. (32) showed that CDO activity increased in response to an injection of cysteine (100 mg/100 g body weight), but the response of CDO to cysteine was not blocked by a simultaneous injection of cycloheximide (0.5 mg/100 g body weight). This result indicates that a mechanism other than protein synthesis may account for the response of CDO to cysteine. No other studies have been conducted on the mechanism of nutritional regula-
tion of CDO. For GCS, numerous studies have been conducted to determine the mechanism of regulation in response to chemotherapeutic agents (34), oxidative stress (24), and hormones (21, 28), but no studies have been conducted that specifically address the mechanism for regulation of GCS in response to changes in dietary protein and methionine. For CSDC, Jerkins et al. (16) showed a decrease in CSDC activity in response to a high dietary protein level, and the change in enzyme activity corresponded to changes in steady-state CSDC protein and mRNA levels.

The purpose of this study was to further evaluate the mechanisms of regulation of hepatic CDO, GCS, and CSDC activities in response to dietary protein or sulfur amino acids. The effects of dietary protein and methionine on CDO and GCS heavy subunit (GCS-HS) concentrations and mRNA levels are reported for the first time, providing more insight into the potential reciprocal regulation of CDO and GCS.

MATERIALS AND METHODS

Animals and Dietary Treatments

Semi-purified diets were prepared as shown in Table 1 to contain various levels of protein and methionine: 100 g casein/kg diet (low protein; LP), 400 g casein/kg diet (moderate protein; MP), 100 g casein + 3 g methionine/kg diet (moderate methionine; LP + MM), or 100 g casein + 10 g methionine/kg diet (high methionine; LP + HM). The LP diet provided 3.2 g of sulfur amino acids; sulfur amino acids were derived solely from casein in the LP diet and from both casein and purified methionine in the other diets. Male Sprague-Dawley rats that weighed ~150 g were purchased from Harlan Sprague Dawley (Indianapolis, IN). Rats were housed individually in stainless steel mesh cages in a room maintained at 20°C and 60–70% humidity with light from 2000 to 0800. Animals were fed a nonpurified diet (RHM 1000, Agway, Syracuse, NY) for 5 days before being assigned to a specific experimental diet. A total of 30 animals were blocked by body weight, and rats within each block were randomly assigned to receive one of the five diets, such that the weight distribution of rats in each treatment group was similar. Five to six animals were assigned to the LP, HP, LP + MM, and LP + HM diets, and 8 rats were assigned to the MP diet; more rats were assigned to the MP group because we previously have observed high variability in hepatic CDO activity among rats fed diets with 20% casein.

Rats had free access to diet and water for the duration of the experiment. Beginning on day 15 of dietary treatment, 7 or 8 rats per day were killed (CO2 anesthesia + decapitation) on each of 4 days. Within assigned blocks, rats were killed in random order. One to two rats per dietary group were killed on each of the 4 days. The care and use of animals were approved by the Cornell University Institutional Animal Care and Use Committee.

The liver was removed, rinsed with ice-cold saline, blotted, and weighed. Approximately 150 mg of liver were homogenized in denaturation solution (TotALLY RNA kit, Ambion, Austin, TX) and then stored at –70°C for later measurement of CDO, GCS-HS, and CSDC mRNAs. The liver was then minced and homogenized in appropriate ice-cold buffers; homogenate was used immediately for enzyme assays or to obtain the soluble fraction, liver homogenate (20%, wt/vol, in 50 mmol/l 2-(N-morpholino)ethanesulfonic acid (MES) buffer, pH 6.0) was centrifuged at 20,000 g for 30 min at 4°C, and the low-speed supernatant was centrifuged at 100,000 g for 60 min at 4°C to obtain high-speed supernatant, which was stored at –70°C until analyses were performed.

Enzyme Assays

CDO and CSDC activities were measured as described previously (5). For assay of GCS activity, minced liver was homogenized in 50 mmol/l N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES) buffer, pH 8.5, to prepare a 20% (wt/vol) homogenate. The activity of GCS was measured by incubating 0.35 or 0.7 ml of liver homogenate (~0.07 or 0.14 g liver) with 1 mmol/l L-cysteine, 20 mmol/l L-glutamate, 10 mmol/l glycine, 0.05 mmol/l bathocuproine disulfonate (BCS), 5 mmol/l MgSO4, 50 mmol/l KCl, 10 mmol/l ATP, 10 mmol/l phosphocreatine, 37.5 U of creatine phosphokinase, and 100 mmol/l HEPES buffer (pH 8.5) in a final volume of 2.5 ml for 15 (0.14 g homogenate) or 30 min (0.07 g homogenate) at 37°C. To terminate the reaction, 0.5 ml of the reaction

Table 1. Composition of semipurified diets containing various levels of protein and methionine

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>LP</th>
<th>MP</th>
<th>HP</th>
<th>LP + MM</th>
<th>LP + HM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin-free casein</td>
<td>100</td>
<td>200</td>
<td>400</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>L-Methionine</td>
<td>3.0</td>
<td>10.0</td>
<td>10.0</td>
<td>10.0</td>
<td>10.0</td>
</tr>
<tr>
<td>Cornstarch</td>
<td>376.5</td>
<td>326.5</td>
<td>226.5</td>
<td>375.0</td>
<td>371.5</td>
</tr>
<tr>
<td>Sucrose</td>
<td>376.5</td>
<td>326.5</td>
<td>226.5</td>
<td>375.0</td>
<td>371.5</td>
</tr>
<tr>
<td>Cellulose</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
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<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
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<tr>
<td>Vitamin mix (AIN 76A)</td>
<td>30</td>
<td>10</td>
<td>10</td>
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<td>10</td>
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<tr>
<td>Mineral mix (AIN 76B)</td>
<td>35</td>
<td>35</td>
<td>35</td>
<td>35</td>
<td>35</td>
</tr>
<tr>
<td>Choline bitartrate</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

Diet was prepared by Dyets (Bethlehem, PA). LP, low protein; MP, moderate protein; HP, high protein; LP + MM, moderate methionine; LP + HM, high methionine. The basal LP diet provided 3.2 g sulfur amino acids (2.8 g Met + 0.4 g Cys) per kg diet. Both MP and LP + MM diets provided –6 g sulfur amino acids, and both HP and LP + HM diets provided ~13 g sulfur amino acids.
mixture was transferred to a tube containing 0.2 ml of 6.2 mol/l perchloric acid, 1.25 mmol/l \( \gamma \)-glutamylglutamate (internal standard for HPLC), 3.75 mol/l BCS, and 3.75 mol/l bathophenanthroline disulfonate. Reactions stopped at time 0 served as blanks. GSH and \( \gamma \)-glutamylcysteine were quantitated by the HPLC method of Fariss and Reed (9) as modified by Stipanuk et al. (27) with GSH as the standard.

Immunoochemical Methods

Sources of antibodies. Rabbit anti-CSDC serum was a gift from Dr. Owen Griffith (Medical College of Wisconsin, Milwaukee, WI). The purified IgG fraction from rabbit anti-CDO serum was a gift from Dr. Yu Hosokawa (National Institute of Health and Nutrition, Tokyo, Japan). Rabbit anti-GCS heavy catalytic subunit serum (GCS-HS) was a gift from Dr. Henry J. Jay Forman (Univ. of Southern California, Los Angeles, CA). The preparation of these antibodies against rat liver CSDC (30), rat liver CDO (12), and a peptide sequence of rat kidney GCS-HS (24) has been reported.

Western blot analysis. Total liver supernatant protein was separated by one-dimensional SDS-PAGE (20) using SDS-polyacrylamide gels (15, 10, and 12% wt/vol polyacrylamide for CDO, GCS-HS, and CSDC analyses, respectively), and the proteins were then electrodusted (29) onto Immobilon-P membranes (Millipore, Medford, MA). Immunoreactive protein was detected by chemiluminescence by use of goat anti-rabbit IgG (Pierce, Rockford, IL) conjugated to horseradish peroxidase (HRP) and the Supersignal CL-HRP substrate system (Pierce, Rockford, IL) with exposure to Kodak X-OMAT XR film. Two-dimensional quantitative densitometric analysis of the regions of interest was performed with Molecular Analyst software (Bio-Rad Laboratories, Hercules, CA). Single bands corresponding to 22.5 kDa for CDO, 74 kDa for GCS-HS, and 54 kDa for CSDC were detected except as noted in results for CDO; apparent molecular masses were consistent with previously published values (17, 23, 31). Relative protein amounts were quantitated using standard curves (pixel density per mm² vs. µg total liver protein loaded) run on each gel; the relative amount of protein was then divided by the actual amount of total protein loaded for each sample. For CSDC and GCS-HS, 5.25 µg and 25 µg for CDO, 74 kDa for GCS-HS, and 54 kDa for CSDC were detected except as noted in results for CDO; apparent molecular masses were consistent with previously published values (17, 23, 31). Relative protein amounts were quantitated using standard curves (pixel density per mm² vs. µg total liver protein loaded) run on each gel; the relative amount of protein was then divided by the actual amount of total protein loaded for each sample. For CSDC and GCS-HS, 5.25 µg and 25 µg, respectively, of total soluble liver protein were loaded per lane; for CDO, 5–210 µg of total soluble liver protein were loaded depending on the treatment group, because the degree of differences among diets was much greater than the linear range of the standard curve. Protein concentration in the liver supernatant samples was determined by the method of Smith et al. (25).

Antibody specificity. For antibody specificity determinations, rabbit serum (Sigma, St. Louis, MO) served as a negative control for anti-CSDC and anti-GCS-HS serum, and purified rabbit serum IgG (11) served as a negative control for anti-CDO serum IgG. In Western blot analyses, duplicate lanes were loaded with samples from each diet group for separation by SDS-PAGE. After transfer of the separated proteins to the membrane, one-half of each membrane was incubated with either anti-CSDC serum, anti-GCS-HS serum, or anti-CDO IgG, and the remaining portion of the membrane was incubated with the corresponding negative rabbit serum control; no bands were detected with control serum or IgG under the conditions used for Western blot analysis.

Immunoprecipitation of all three enzyme activities was also performed to confirm the specificity of the respective antibodies for CDO, GCS-HS, and CSDC. Briefly, liver was incubated with graded levels of anti-CSDC serum, anti-GCS serum, anti-CDO IgG, or the corresponding negative rabbit serum control. The antibody-antigen complex was precipitated using protein A agarose and protein G sepharose. Enzyme activity corresponding to the antibody and one of the other two enzyme activities (negative control) were measured in the remaining supernatant. Immunoprecipitation of CDO activity was of particular interest, because the relationship of purified protein used to raise the antibody to the physiologically active CDO had not been evaluated. A dose-dependent immunoprecipitation was observed with antibodies to CDO and CSDC, and the immunoprecipitation was specific. The extent of precipitation was ~80% for CDO and 100% for CSDC. GCS activity was decreased by ~50% when liver supernatant was incubated with the highest amount of anti-GCS-HS serum; no additional decrease in GCS activity occurred with greater amounts of anti-GCS-HS serum. Because a 19 amino acid-peptide sequence of GCS-HS, rather than the intact protein, was used to raise antibodies to GCS-HS, the lower immunoprecipitation response is probably related to the limited sites at which the anti-GCS-HS could bind to GCS.

Northern and dot-blot analyses. Preparation of cDNA probes. An EcoRI 1-cut cDNA for CDO (12) was a gift of Dr. Yu Hosokawa and Nobuyo Tsuboyama (National Institute of Health and Nutrition, Tokyo, Japan). Complementary DNA probes corresponding to bp 459–717 of rat liver CDO (12), bp 390–653 of rat liver GCS-HS (33), and bp 74–310 of rat kidney CSDC (19) were synthesized using PCR (CDO) or RT-PCR (GCS-HS and CSDC). Each DNA was cloned into pBluescript SK+, and the sequence of the cloned DNA was verified by DNA Services (Cornell University, Ithaca, NY). Cloned cDNA was used to prepare labeled probes by PCR and labeling with [32P]dCTP using random priming (CDO and CSDC) or the reverse primer (GCS-HS). Deka probe template-actin-mouse (Ambion, Austin, TX) was labeled with [32P]dCTP using random primers and used as an internal standard for Northern and dot-blot analyses of CDO, CSDL, and GCS-HS mRNAs.

Isolation of total RNA and Northern blot and dot-blot hybridization analyses. Total RNA was isolated from liver (8) using the TOTALLY RNA kit (Ambion). Northern blot analysis was done as described by Brown (7) with electrophoresis of total liver RNA (10 µg) on a 1% (wt/vol) agarose-formaldehyde gel and blotting onto a Magna Graph nylon membrane (Micron Separations, Westboro, MA). Membranes were prehybridized using herring sperm DNA and then hybridized with one of the [32P]labeled cDNA probes (0.034 MBq/ml buffer). After hybridization, membranes were washed twice in 2× saline-sodium phosphate-EDTA buffer (SSPE) with 0.2% (wt/vol) SDS for 30 min at room temperature and twice in 0.1× SSPE at 60°C for 15 min and then autoradiographed using Kodak X-OMAT AR film. Probes were stripped from the membrane by boiling the membrane in 0.1× SSPE with 1% (wt/vol) SDS between hybridization with the various probes. Northern analysis yielded single bands for CDO mRNA of ~1.7 kb, for GCS-HS mRNA of ~4.1 kb, and for CSDC mRNA of ~2.5 kb; the sizes of the mRNAs were consistent with previously published values (12, 14, 19).

For dot blot analysis and quantification of relative amounts of enzyme mRNA, 12 µg of total RNA from each of the 30 animals were applied to a Magna Graph nylon membrane using a microsample filtration manifold (Minifold, Schleicher and Schuell, Keene, NH). The membranes were hybridized with the [32P]labeled cDNA probes as described above. Results were quantified using the Bio-Rad GS 363 Phosphorescence Imaging System (Bio-Rad Laboratories, Melville, NY) and the Molecular Analyst program (Bio-Rad Laboratories, Hercules,
CA). A standard curve was generated on each membrane by loading 2.5–17.5 μg of pooled total RNA. Units for the relative amount of mRNA for each enzyme and actin were calculated using a standard curve of pixel density per squared millimeter vs. the amount of total RNA loaded. Relative enzyme mRNA amount was calculated from the standard curve and was corrected for loading by dividing the relative enzyme mRNA amount by the relative actin mRNA amount.

Statistics

Data were analyzed by analysis of variance (Minitab 8.1.1, State College, PA) and Tukey’s or Tukey-Kramer’s W-procedure (26). Correlation coefficients were calculated using Microsoft Excel 5.0 (Microsoft, Cambridge, MA). Differences were considered significant at P ≤ 0.05.

RESULTS

Weight Gain, Food Intake, and Hepatic Levels of Protein, Taurine, and GSH

The LP or basal diet contained (wt/wt) 10% casein, which provided ~0.3% sulfur amino acids; these levels are less than the recommended protein and sulfur amino acid intakes for growing rats, which are 12% protein and 0.6% sulfur amino acids (22). Although the LP diet was deficient in protein, with sulfur amino acids being first limiting, it did support a reasonable rate of weight gain, an average of 4.1 g/day. As expected, the weight gain was greater in rats fed diets supplemented with additional casein or with additional methionine, with casein supplementation allowing greater weight gain than methionine alone (at equivalent amounts of total sulfur amino acids). The improvement in weight gain was due to the dietary improvement and not to greater food intake, as shown in Table 2.

The hepatic protein concentration (mg protein/g liver) in rats fed the LP diet was similar to that for rats fed the other diets, and most results are expressed on the basis of liver protein. The relative liver weight (g liver/100 g body weight) was similar for rats fed the LP, MP, HP, and LP + MM diets but was somewhat elevated in rats fed the LP + HM diet.

The hepatic taurine concentration (nmol/mg protein) was significantly higher (P ≤ 0.05) in rats fed diets supplemented with either protein or methionine than in rats fed the LP diet; a dose-response relationship was observed for both protein and methionine supplementation. At the moderate level of supplementation, supplementation with methionine resulted in greater hepatic taurine levels than did supplementation with protein (LP + MM vs. MP). The hepatic GSH level (nmol/mg protein) was greater in rats fed diets supplemented with methionine or protein than in rats fed the basal LP diet. Among rats fed the supplemented diets, those fed the LP + HM diet had significantly higher (P ≤ 0.05) hepatic GSH levels than did rats fed the MP, HP, or LP + MM diets. Various factors that determine hepatic GSH and taurine levels in response to protein or sulfur amino acid intake have been reviewed previously (4).

CDO, GCS, and CSDC Activities

Consistent with previous observations, CDO activity differed in a dose-dependent manner in response to the amount of dietary sulfur amino acids, either as a component of protein or as methionine alone. As shown in Fig. 2, the CDO activity in liver of rats fed the MP and HP diets was 14 and 29 times, respectively, the level of activity in rats fed the LP diet. The pattern of CDO activity observed with methionine supplementation was similar to that observed with protein supplementation at equisulfur levels; hepatic CDO activity in rats fed the LP + MM and LP + HM diets was 18 and 35 times, respectively, the level of activity in rats fed the LP diet. With both protein and methionine supplementation, the magnitude of the degree of increase in CDO activity was greater for the step between LP and MP (14-fold) or LP and LP + HM (17-fold) than for the step between MP and HP (1-fold) or LP + MM and LP + HM (1-fold). Thus marked increases in CDO activity may occur when either the protein or sulfur amino acid intake is increased from below to at or somewhat above the requirement level.

Hepatic GCS activity differed in a stepwise manner in rats fed diets with different levels of either dietary protein or methionine. The level of hepatic GCS activity in rats fed the MP and HP diets was 50 and 19%, respectively, of the activity in rats fed the LP diet. Mean levels of GCS activity in rats fed the LP + MM and LP + HM diets were 70 and 39%, respectively, of the activity in rats fed the LP diet. Thus, although lower GCS activity consistently was observed in rats fed

Table 2. Effects of diet on food intake and weight gain of rats and on protein, taurine, and GSH concentrations of rat liver.

<table>
<thead>
<tr>
<th></th>
<th>LP n = 6</th>
<th>MP n = 8</th>
<th>HP n = 5</th>
<th>LP + MM n = 5</th>
<th>LP + HM n = 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average daily diet consumption, g</td>
<td>25.8 ± 2.0</td>
<td>22.6 ± 0.6</td>
<td>22.0 ± 0.5</td>
<td>23.4 ± 0.5</td>
<td>25.8 ± 1.5</td>
</tr>
<tr>
<td>Average daily weight gain, g</td>
<td>4.1 ± 0.6</td>
<td>6.1 ± 0.3</td>
<td>6.0 ± 0.4b</td>
<td>5.0 ± 0.3ab</td>
<td>5.4 ± 0.2b</td>
</tr>
<tr>
<td>Body weight at end of feeding period, g</td>
<td>281 ± 9.1</td>
<td>321 ± 4.3</td>
<td>321 ± 5.8</td>
<td>313 ± 5.1b</td>
<td>312 ± 2.7b</td>
</tr>
<tr>
<td>Liver weight at end of feeding period, g</td>
<td>28.6 ± 0.7</td>
<td>12.8 ± 0.4ab</td>
<td>12.9 ± 0.4ab</td>
<td>12.8 ± 0.6ab</td>
<td>14.3 ± 0.4</td>
</tr>
<tr>
<td>Relative liver weight, g/100 g body wt</td>
<td>38.4 ± 0.4</td>
<td>4.0 ± 0.2ab</td>
<td>4.0 ± 0.2ab</td>
<td>4.0 ± 0.4ab</td>
<td>4.6 ± 0.2a</td>
</tr>
<tr>
<td>Hepatic taurine, nmol/mg protein</td>
<td>3.3 ± 0.3</td>
<td>6.8 ± 0.6ab</td>
<td>90.1 ± 90.0</td>
<td>18.9 ± 5.3c</td>
<td>59.3 ± 10.2d</td>
</tr>
<tr>
<td>Hepatic GSH, nmol/mg protein</td>
<td>13.3 ± 1.5</td>
<td>22.3 ± 1.5b</td>
<td>22.8 ± 1.0b</td>
<td>21.8 ± 1.6b</td>
<td>32.1 ± 1.6c</td>
</tr>
<tr>
<td>Liver protein, mg/g liver</td>
<td>210.2 ± 3.9</td>
<td>215.2 ± 4.2</td>
<td>223.2 ± 1.9</td>
<td>204.7 ± 4.2</td>
<td>200.5 ± 5.1</td>
</tr>
</tbody>
</table>

Values are means ± SE. GSH, glutathione. Within a row, values with different superscripts are significantly different (P ≤ 0.05) by ANOVA and Tukey-Kramer’s W-procedure. Data for taurine were transformed to log10 before statistical analysis.
either excess protein or purified methionine, the magnitude of change in activity tended to be less marked when sulfur amino acids were provided as methionine alone than when they were provided as a component of dietary protein.

The observed effects of dietary protein or methionine on CSDC activity were generally similar to the effects observed in other studies (2, 4–6). Hepatic CSDC activity in rats fed the LP and MP diets was similar (P > 0.05), with activity in rats fed the MP diet tending to be slightly greater than activity in rats fed the LP diet. The only dietary treatment that resulted in a significant difference in hepatic CSDC activity relative to that of rats fed the LP diet was the HP diet; hepatic CSDC activity in rats fed the HP diet was 52% of the activity in liver of rats fed the LP diet. Hepatic CSDC activity in rats fed the LP+MM and LP+HM diets was 95 and 77%, respectively, of activity in rats fed the LP diet.

CDO, GCS-HS, and CSDC Protein Concentrations

The relative CDO protein levels in liver of rats fed the MP and HP diets were 10.5 and 44 times, respectively, the level of CDO protein observed in rats fed the LP diet (Fig. 3). CDO concentrations in rats fed the LP+MM and LP+HM diets were 13 and 26 times, respectively, the level observed in rats fed the LP diet.

GCS-HS concentrations were lower in liver of rats fed higher levels of dietary protein, with the level of hepatic GCS-HS in rats fed the MP and HP diets being 81 and 67%, respectively, of that observed in rats fed the LP diet. The concentration of GCS-HS in liver of rats fed the LP+MM and LP+HM diets was 73 and 70%, respectively, as much as that observed in liver of rats fed the LP diet.

The CSDC concentration in liver of rats fed the MP and HP diets was 85 and 50%, respectively, of that observed in rats fed the LP diet. In rats fed the LP+MM
and LP+HM diets, CSDC protein levels were 91 and 90%, respectively, of the concentration observed in rats fed the LP diet.

In Western blot analysis of CDO, two distinct bands were detected by the anti-CDO IgG in liver samples from rats fed the HP and LP+HM diets (Fig. 4). A CDO band with an estimated molecular mass of 25.5 kDa was observed in liver of all dietary treatment groups. An additional band with a molecular mass of 23.5 kDa was observed only in liver of rats fed the HP and LP+HM diets. This lower band was not detected when the amounts of protein from the other dietary groups that were loaded onto the gel were increased up to 6 times the amount loaded for the HP and LP+HM groups. The molecular mass for the upper band is slightly higher than the molecular mass (23 kDa) calculated from the amino acid sequence (12) or the molecular mass (22 kDa) estimated by SDS-PAGE of purified (largely inactive) CDO (31). However, the apparent molecular mass for the lower band is in close agreement with these previously reported molecular masses. It was not possible to quantitate the density of the two bands separately, and the quantitative results for relative CDO protein reported for the HP and LP+HM groups in Fig. 3 include both bands.

CDO, GCS-HS, and CSDC mRNA Levels

No marked differences were observed in steady-state hepatic CDO mRNA levels among the five dietary groups (Fig. 5). Because two CDO species were detected by Western blot analysis, RT-PCR with primers that spanned the open reading frame was used to determine whether more than one CDO mRNA transcript existed in livers of rats fed the HP and LP+HM diets. No evidence for a second CDO mRNA species was obtained.

Hepatic steady-state GCS-HS mRNA levels also showed a dose-dependent response to dietary protein or methionine level. The relative abundance of GCS-HS mRNA in liver of rats fed the MP and HP diets was 70 and 45%, respectively, of that observed in rats fed the LP diet. Steady-state GCS-HS mRNA abundance in rats fed the LP+MM and LP+HM diets was 63 and 49%, respectively, of that observed in rats fed the LP diet.

Hepatic steady-state CSDC mRNA abundance values in rats fed the HP, LP+MM, and LP+HM diets were 28, 70, and 62%, respectively, of those observed in rats fed the LP diet, whereas CSDC mRNA levels in liver of rats fed the MP diet were 106% of levels observed in rats fed the LP diet. Compared with the abundance of CSDC mRNA in the LP group, only the HP group had a significantly different (P ≤ 0.05) amount.

**DISCUSSION**

The multiples of change in enzyme activity, enzyme concentration, and mRNA concentration observed in this study are summarized for CDO, GCS-HS, and CSDC in Table 3. Ratios of enzyme activity to enzyme concentration (activity state) and of enzyme concentration to enzyme mRNA are also shown.

Regulation of CDO

The similarity of the large degree differences in CDO activity and CDO concentration (protein) suggests that the higher CDO activity observed in rats fed diets supplemented with dietary protein or methionine is due predominantly to an increase in the steady-state level of CDO rather than to an increase in specific activity of the enzyme. Marked similar increases in both steady-state CDO activity (13.5- and 16-fold for MP and LP+MM, respectively) and CDO protein (9.5- and 12-fold for MP and LP+MM, respectively) were observed in animals fed diets with either 10% additional casein (MP vs. LP) or 0.3% supplemental methionine (LP-MM vs. LP). Thus the greatest changes in CDO activity occurred in the range of protein and sulfur amino acid requirements, were due to changes in CDO concentration, and could be accounted for by the sulfur amino acid content of the diet.

With further increases in dietary protein (HP) or methionine (LP+HM), CDO activity doubled (1-fold increase) and CDO protein increased by 3.2-fold (HP) or 1-fold (LP+HM) relative to the MP and LP+MM groups, respectively. Clearly the greater degree of change in CDO protein relative to activity that was noted only for the HP group indicates an apparent relative specific activity of CDO in the HP group that was about one-half of that in the other supplemented groups; this suggests that some additional regulation may result from changes in the CDO activity state at high dietary protein levels.

CDO activity in rats fed the HP and LP+HM diets could also be affected by the appearance of the second immunoreactive species of CDO, which was observed only in liver of rats fed these two diets with the highest level of protein or methionine. The presence of this second CDO band has been confirmed in another study in our laboratory in which rats were fed a crystalline amino acid diet designed to simulate a 40% protein diet or a high-methionine diet similar to the one fed in this study (D. L. Bela, C. Hahn, and M. H. Stipanuk, unpublished observations). Therefore, the presence of the second CDO species appears to be directly related to the composition of the diet and to occur only when relatively high levels of protein or sulfur amino acids are fed. The relative activity of the two CDO bands is not known, and it is not clear whether the presence of the second band is related to the apparent lower CDO specific activity observed in the HP group.

![Fig. 4. Western blot of CDO in liver of rats fed diets that varied in protein and methionine levels. Amount of soluble protein loaded and dietary treatment group from which pooled sample was obtained are indicated above bands.](http://ajpendo.physiology.org/ by 10.220.33.6 on November 6, 2017)
The nature of the difference in the two protein bands detected by anti-CDO IgG in rats fed the HP and LP1 HM diets has not been fully explored. The possibility that the rat CDO message may be alternatively spliced within the open reading frame, resulting in translation of two CDO products, seems unlikely on the basis of our preliminary efforts to identify more than one CDO mRNA transcript. The two CDO species may differ in some type of posttranslational modification that alters the apparent molecular mass of the CDO protein, or the second CDO species may represent a CDO degradation product that is stabilized in liver of rats fed high protein or methionine diets. It also is possible that the two CDO bands represent products of different genes, because the purified protein used to raise the anti-CDO IgG contained a trace amount of a lower apparent molecular mass protein that also had CDO activity (Hosokawa, unpublished observations). Although additional studies are necessary to define the nature of the two CDO species, it is clear that regulation of CDO activity in response to dietary protein or methionine over the range of usual protein intakes predominantly occurs at the level of enzyme concentration and does not involve the second CDO species.

Hepatic CDO mRNA abundance was unaffected by the dietary treatments, ruling out regulation by a pretranslational mechanism. In particular, it should be noted that the CDO mRNA transcript was abundant in liver of rats fed the LP diet, but the level of CDO (both CDO protein and CDO activity) was extremely low. This suggests that, in rats fed a low-protein diet, either the CDO mRNA is translated very slowly or the protein product is rapidly degraded. Regulation of CDO concentration must occur either translationally or posttranslationally, predominantly as a result of either an increase in the rate of translation of CDO mRNA or a decrease in the rate of degradation of CDO protein. The earlier work of Yamaguchi et al. (32) and Hosokawa et al. (13), which demonstrated an increase in the CDO half-life in liver of rats injected with cysteine, lends support to the hypothesis that regulation of CDO activity in response to sulfur amino acids occurs at the level of stabilization of CDO protein.

This is the first study in which the effects of dietary protein and sulfur amino acid level on CDO concentra-

Table 3. Summary of degree changes in enzyme activity, protein concentration, and mRNA concentration for CDO, GCS, and CSDC with protein or methionine supplementation

<table>
<thead>
<tr>
<th></th>
<th>Activity</th>
<th>Protein</th>
<th>mRNA</th>
<th>Activity/Protein</th>
<th>Protein/ mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDO</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LP</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>MP</td>
<td>15.1</td>
<td>11.0</td>
<td>1.0</td>
<td>1.5</td>
<td>10</td>
</tr>
<tr>
<td>HP</td>
<td>30.44</td>
<td>12.1</td>
<td>0.7</td>
<td>36.0</td>
<td></td>
</tr>
<tr>
<td>LP + LM</td>
<td>18.13</td>
<td>12.1</td>
<td>1.4</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>LP + HM</td>
<td>35.26</td>
<td>11.1</td>
<td>1.4</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>GCS-HS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LP</td>
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<td>1.0</td>
</tr>
<tr>
<td>MP</td>
<td>0.55</td>
<td>0.81</td>
<td>0.70</td>
<td>0.68</td>
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</tr>
<tr>
<td>HP</td>
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<td>0.67</td>
<td>0.45</td>
<td>0.39</td>
<td>1.5</td>
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<tr>
<td>LP + MM</td>
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<td>0.63</td>
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<tr>
<td>LP + HM</td>
<td>0.47</td>
<td>0.70</td>
<td>0.49</td>
<td>0.67</td>
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<td>CSDC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LP</td>
<td>1.00</td>
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<td>1.0</td>
<td>1.0</td>
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<tr>
<td>MP</td>
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<tr>
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<td>0.50</td>
<td>0.28</td>
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<tr>
<td>LP + MM</td>
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<td>0.91</td>
<td>0.70</td>
<td>1.0</td>
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<tr>
<td>LP + HM</td>
<td>0.77</td>
<td>0.90</td>
<td>0.62</td>
<td>0.9</td>
<td>1.4</td>
</tr>
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</table>

Values for cysteine dioxygenase (CDO), γ-glutamylcysteine synthetase heavy subunit (GCS-HS), and cysteine-sulfinate decarboxylase (CSDC) activity, protein, and mRNA are means for each group (see Figs. 2, 3, and 5) divided by the mean for LP group.
Transcription and CDO mRNA level have been measured. Our data clearly indicate that upregulation of CDO in response to dietary protein does not occur by a pretranslational mechanism, but by a translational or posttranslational mechanism that results in changes in CDO concentration in response to the sulfur amino acid intake.

Regulation of GCS

Supplementation of the low-protein basal diet with either protein or methionine resulted in lower levels of hepatic GCS activity and of GCS-HS protein and GCS-HS mRNA. The changes in GCS activity, GCS-HS concentration, and GCS-HS mRNA were modest, with the highest GCS activity being only four times the lowest observed activity. The effects of protein or methionine supplementation on GCS activity, as well as on GCS-HS protein and GCS-HS mRNA concentrations, occurred in a dose-related manner over the range of intakes studied (Table 3).

Lower GCS-HS concentrations were associated with lower GCS-HS mRNA abundance, and thus hepatic GCS activity in response to dietary protein seems to be regulated pretranslationally. Thus regulation of GCS-HS gene expression in response to protein may be transcriptionally regulated, as has been shown for the response to changes in insulin, hydrocortisone, several chemotherapeutic agents, and oxidative stress (21, 24, 34). The lower abundance of GCS-HS mRNA could also be the result of decreased stability of the GCS-HS mRNA. The steady-state GCS-HS protein and GCS-HS mRNA levels were highly correlated ($r^2 = 0.97$), and the correlation coefficient did not change when the data were analyzed separately for protein and methionine effects. Thus our results indicate that the pretranslational regulation of GCS-HS is a response to the sulfur amino acid content of the diet.

Interestingly, the results summarized in Table 3 also suggest that GCS activity is regulated posttranslationaly, i.e., that the activity state of GCS-HS is affected by diet. By making comparisons at approximately equal intakes of sulfur amino acids, one can see that GCS protein levels were similarly affected by protein or methionine supplementation, whereas both GCS-HS activity and GCS-HS activity state (GCS activity/GCS-HS concentration) consistently were lower in liver of rats fed protein-supplemented diets than in liver of rats fed methionine-supplemented diets. These changes in GCS-HS activity state thus seem to result from changes in protein intake and cannot be explained fully by sulfur amino acid intake alone. The differential effects of sulfur amino acids vs. protein on GCS activity state were evidenced by a stronger correlation between GCS activity and total immunodetected GCS-HS protein levels when the relationship was analyzed separately for response to protein ($r^2 = 0.97$) and response to methionine ($r^2 = 0.97$) than when data were combined ($r^2 = 0.75$).

One possible explanation for a decrease in the activity state of the GCS-HS protein is that the enzyme may be phosphorylated in response to a high-protein or high-methionine diet. Phosphorylation of GCS-HS has been suggested as a mechanism of downregulation of hepatic GCS activity in response to glucagon and phenylephrine (28). Another possible explanation is that changes in dietary protein level may affect the concentration of the light subunit of GCS (GCS-LS) or its interaction with GCS-HS. GCS-LS is required for maximal GCS activity, and it contributes to regulation of GCS-HS by affecting GCS-HS interactions with substrate at the active site (14).

Although less dramatic and requiring confirmation, our results also suggest a possible effect of diet on the ratio of GCS-HS protein to GCS-HS mRNA. Higher ratios of GCS-HS protein to GCS-HS mRNA were observed in response to either higher protein or higher methionine intakes, with the response following a dose-dependent pattern. An elevated ratio of GCS-HS protein relative to GCS-HS mRNA could result from a higher rate of translation of GCS-HS mRNA or a lower rate of degradation of GCS-HS protein; this latter response is clearly in the opposite direction from the overall changes in GCS activity, GCS-HS concentration, and GCS-HS mRNA abundance and, if true, would limit the magnitude of the downregulation of GCS activity in response to increases in sulfur amino acid intake.

Although GSH synthesis has been widely studied, this is the first study in which the effects of dietary protein and sulfur amino acid level on GCS-HS concentration and mRNA level have been measured. Downregulation of GCS activity in response to dietary protein or methionine occurred as a result of pretranslational regulation of the GCS-HS mRNA level, with the changes in GCS-HS mRNA abundance clearly being a response to sulfur amino acid intake. Regulation of GCS activity in response to dietary protein, and to a lesser extent methionine, also occurred by a posttranslational mechanism.

Regulation of CSDC

Hepatic CSDC activity was significantly different only in the liver of rats fed the HP diet compared with that in rats fed the basal LP diet. As summarized in Table 3, hepatic CSDC activity, CSDC protein, and CSDC mRNA levels in rats fed the HP diet were 52, 50, and 28%, respectively, of the corresponding values for rats fed the LP diet. These changes are consistent with regulation of CSDC via changes in CSDC mRNA abundance. Our findings of changes in CSDC protein and mRNA concentrations are consistent with those of Jerkis et al. (16) who reported decreases in CSDC activity in response to a high dietary protein level, with the change in enzyme activity corresponding to the changes in CSDC protein and mRNA levels. Thus it appears that downregulation of CSDC in response to a high-protein diet occurs pretranslationally as a result of either a lower rate of CSDC gene transcription or an increased rate of degradation of CSDC mRNA.

The ratio of CSDC activity to immunodetected CSDC protein suggested that the activity state was similar in liver of rats fed the LP and HP diets, but the ratio of...
CSDC protein to mRNA was 80% higher in rats fed the HP diet than in rats fed the LP diet, suggesting a possible increase in the rate of CSDC mRNA translation or increased stability of CSDC protein. If some type of translational or posttranslational upregulation of CSDC concentration occurs in addition to the pretranslational downregulation of CSDC activity, the increase would limit the magnitude of the overall downregulation of CSDC activity in response to dietary protein level. This apparent trend is similar to that observed and discussed above for GCS-HS, but our present data should be viewed as only suggestive of such a mechanism and in need of further study.

Roles of CDO, GCS, and GSDC in the Regulation of Hepatic Cysteine Metabolism

Changes in regulation of CDO and GCS occur over dietary protein or sulfur amino acid levels that encompass the requirement levels for growing rats (22) and appear to play an important role in determining the flux between GSH synthesis and cysteine catabolism (3–6). CSDC was less responsive to methionine than to protein at equisulfur levels, as has been observed in other studies (3, 4, 15). Decreases in CSDC activity occurred at dietary protein levels at or above 25% casein, which are clearly in excess of the protein requirement. Overall, it seems that, in contrast to regulation of CDO and GCS, regulation of CSDC is not a major means of regulation of sulfur amino acid catabolism within the range of typical protein intakes.

The relative degree changes in enzyme activity were much smaller for GCS and CSDC than for CDO; the ratio of highest to lowest activities observed was 35 for CDO, 4 for GCS, and 2 for CSDC. The smaller magnitude of change for GCS most likely allows for sustained GSH synthesis regardless of fluctuations in sulfur amino acid intake, whereas the rate of catabolism of cysteine can vary markedly in response to changes in sulfur amino acid intake. The maintenance of high CSDC activity over the range of typical protein intakes also may protect taurine synthesis compared with cysteine catabolism to sulfate. The roles of these enzymes in the regulation of cysteine catabolism have been documented in a number of previous studies (3–6). The combined effects of changes in sulfur amino acid intake and in CDO and GCS activities are reflected in the hepatic GSH and taurine concentrations reported in Table 2.

Elevated levels of cysteine have been shown to be both cytotoxic and neurotoxic, and increased levels of homocysteine, a precursor of cysteine in the methionine transsulfuration pathway, have been associated with increased risk for cardiovascular disease and with the occurrence of neural tube defects. The ~10-fold greater hepatic CDO activity that occurs in animals fed 20% compared with 10% dietary protein would provide a robust system for prevention of potential damage to the cell by rapid removal of excess sulfur amino acid metabolites. This may be a system that is able to respond rapidly to changes in cellular cysteine concentrations, because it is not dependent on changes in gene transcription.

Role of Sulfur Amino Acids vs. Protein in Effecting Changes in CDO, GCS, and CSDC

Comparison of the effects of dietary protein or methionine at equisulfur levels indicates that the pretranslational regulation of GCS-HS and the translational or posttranslational regulation of CDO were responses to sulfur amino acid intake, whereas the pretranslational regulation of CSDC activity was significant for rats fed the highest level of dietary protein but not methionine. The posttranslational downregulation of GCS-HS activity was stronger in response to protein intake but also occurred with methionine supplementation.

Although reciprocal changes in CDO and GCS in response to sulfur amino acid intake in the range of the requirement do occur consistently, the results of this study indicate that the molecular mechanisms involved in bringing about differences in steady-state CDO and GCS activities largely appear to occur at different points. Nevertheless, regulation of GCS and CDO activities in response to sulfur amino acid intake may occur in response to the same intracellular effector or mediator. Because these enzymes are rate limiting for either cysteine catabolism or GSH synthesis from cysteine, the intracellular cysteine concentration, the concentration of a sulfur amino acid metabolite, or a secondary signal affected by changes in sulfur amino acid availability could serve as the cellular effector(s) for the regulation of these two enzymes. Clearly, both CDO concentration and GCS-HS mRNA concentration changed in response to total sulfur amino acid intake. Further studies are required to elucidate the specific mechanisms, the role of each mechanism, and the specific cellular effector molecule(s) that result in these changes in cysteine metabolism.

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