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Amino acid sequence of rat kidney glutathione synthetase

(inborn errors of metabolism/glycoprotein/cloning/primary structure)

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ABSTRACT **Glutathione (GSH) synthetase [γ-L-glutamyl-L-cysteine:glycine ligase (ADP-forming), EC 6.3.2.3], an enzyme present in almost all cells, catalyzes the ATP-dependent synthesis of GSH from γ-L-glutamyl-L-cysteine and glycine. Highly purified preparations of the enzyme have been obtained from rat kidney and several lower forms. The rat kidney enzyme (M_r, 118,000), which contains ≈2% carbohydrate, is composed of two apparently identical subunits. The cDNA encoding rat kidney GSH synthetase was isolated from a rat kidney λgt11 cDNA library by immunoscreening with an antibody prepared against the isolated enzyme. The cDNA contains 1905 nucleotides and an open reading frame of 1422 nucleotides coding for 474 amino acids. The cDNA has a 3′ untranslated region of 439 nucleotides, which includes a poly(A) tail. The deduced amino acid sequence (M_r, 52,344) contains all five of the peptide sequences that were independently determined by Edman degradation. The cDNA was expressed in *Escherichia coli*. The amino acid sequence of the rat kidney enzyme has no significant similarity to that of the enzyme from *E. coli* and shows some similarity to those deduced for the yeast and frog enzymes. Knowledge of this amino acid sequence is expected to facilitate elucidation of the sequence of the corresponding human enzyme and to lead to studies on the biochemical mechanisms involved in human GSH synthetase deficiency as well as to development of improved methods for prenatal diagnosis of these inborn diseases.**

The important biochemical functions of glutathione (GSH) have been considered in a number of recent reviews and at several conferences (1–4). GSH synthesis takes place within almost all animal cells and in those of many plants and microorganisms. The two enzymes required for synthesis of this tripeptide (γ-glutamylcysteine synthetase and GSH synthetase) have been isolated from a number of different sources (2, 5–7). γ-Glutamylcysteine synthetase, which catalyzes the first and rate-limiting step of GSH synthesis (reaction **1**), is feedback inhibited by GSH (8, 9).

$$\text{L-Glutamate + L-cysteine + ATP} \xrightleftharpoons[\text{synthetase}]{\gamma\text{-glu-cySH}} \text{L-glutamyl-L-cysteine + ADP + P}_i. \quad [1]$$

The amino acid sequences of the two separately coded proteins that comprise the two subunits of this enzyme in mammalian tissues have been deduced (10, 11). This enzyme, which differs substantially in subunit structure and amino acid sequence from bacterial γ-glutamylcysteine synthetase, has been the subject of several studies in this laboratory (8–14).

The enzyme that catalyzes the synthesis of GSH from γ-glutamylcysteine and glycine [GSH synthetase; γ-L-glutamyl-L-cysteine:glycine ligase (ADP-forming), EC 6.3.2.3] (reaction **2**)

$$\text{L-γ-Glutamyl-L-cysteine + glycine + ATP} \xrightleftharpoons[\text{synthetase}]{\text{GSH}} \text{GSH + ADP + P}_i \quad [2]$$

has also been purified from several biological sources (7), but the primary structure of the mammalian enzyme is not yet known. Some data on the amino acid sequences of the GSH synthetases of lower forms [e.g., bacteria, yeast (see below)] are available; there are major differences between certain properties of these enzymes and those of the rat kidney enzyme, which are given here.

Knowledge of the amino acid sequence of mammalian GSH synthetase is essential for further studies on the structure, mechanism of action, and physiological function of this enzyme. Such information will be of importance in understanding the biochemical mechanisms associated with GSH synthetase deficiency in humans. Two general types of such deficiency have been observed (15). In one, an unstable form of GSH synthetase is expressed, leading to an apparently selective deficiency of GSH in the erythrocyte. In another type of GSH synthetase deficiency, dramatic metabolic consequences, potentially fatal, occur as a result of overproduction of 5-oxoproline, which leads to severe metabolic acidosis. In this condition, there is overproduction of γ-glutamylcysteine, whose synthesis is not feedback inhibited because of the low levels of GSH and possibly because there is induction of γ-glutamylcysteine synthetase. γ-Glutamylcysteine is converted by the action of γ-glutamylcyclotransferase to cysteine and 5-oxoproline. Cysteine is used by γ-glutamylcysteine synthetase (in a futile cycle), and 5-oxoproline accumulates in amounts that exceed the capacity of 5-oxoprolinase to convert it to glutamate. This leads to substantial accumulation of 5-oxoproline and to its urinary excretion in amounts that may be as high as 30 g/day (normally <0.14 g/day) (15).

In the present work we cloned the GSH synthetase of rat kidney and deduced its amino acid sequence and certain other properties.*

EXPERIMENTAL PROCEDURES

GSH Synthetase Activity. This enzymatic activity was determined spectrophotometrically by following the formation of ADP using a coupled assay with pyruvate kinase and lactate dehydrogenase (13). The reaction mixture (1 ml) contained Tris·HCl buffer (100 mM; pH 8.2), γ-L-glutamyl-L-α-aminobutyrate (2 mM), glycine (10 mM), magnesium chloride (20 mM), disodium ATP (5 mM), sodium phospho*enol*pyruvate (2 mM), potassium chloride (150 mM), NADH (2 mM), pyruvate kinase (5 units), and lactate dehydrogenase (10 units); the

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Abbreviation: GSH, glutathione.

*The sequence reported in this paper has been deposited in the GenBank data base (accession no. L38615).

reaction was initiated by addition of the enzyme sample. The rate of decrease in the absorbance at 340 nm was followed at 37°C. A unit of enzyme activity is defined as the amount that catalyzes the formation of 1 μmol of product per h.

Purification of GSH Synthetase. GSH synthetase was isolated from rat kidney by ammonium sulfate fractionation, heat treatment, DE52, and Sephadex G-150 column chromatography as described by Oppenheimer *et al.* (16). The enzyme was further processed by chromatography on a phenyl-5PW HPLC column (Bio-Rad) equilibrated with 50 mM imidazole HCl buffer (pH 7.4) containing 1 mM EDTA and 0.5 M ammonium sulfate (starting buffer). The column was eluted with a linear gradient established between the starting buffer and an imidazole HCl buffer (50 mM; pH 7.4) containing 1 mM EDTA for 40 min. Fractions of 1 ml were collected. The fractions containing enzyme activity were pooled and dialyzed against 50 mM imidazole HCl buffer (pH 7.4). The specific activity was 780 units/mg. SDS/PAGE analysis showed apparent homogeneity (>99%) and a M_r of 55,000.

Preparation of Antibody Against Rat Kidney GSH Synthetase. The synthetase (0.1 mg dissolved in 1 ml of phosphate-buffered saline) was mixed vigorously with 1 ml of complete Freund's adjuvant until the solution became very viscous. Anesthetized New Zealand White rabbits (4 lb; 1 lb = 0.453 kg) were injected intradermally with 10 portions (0.2 ml each) of the emulsion. The animals were given a second dose of a mixture containing the protein (0.1 mg) mixed with the same volume of incomplete Freund's adjuvant 6 weeks later. The titer of the antiserum was monitored by ELISA. Since the antibody obtained apparently reacts with *Escherichia coli* protein, the antiserum was preabsorbed with the *E. coli* protein by incubating it with nitrocellulose that had been saturated with *E. coli* total protein before immunoscreening.

Peptide Sequence Analysis. The protein was digested with trypsin and with endopeptidase LysC as described (17, 18). Rat kidney GSH synthetase (1 mg) was subjected to SDS/PAGE and electrotransferred to a poly(vinylidene difluoride) membrane. The membrane was stained with amido black and the protein band with a M_r of 55,000 was cut off the membrane and blotted with 0.5% polyvinylpyrrolidone 40. The membrane was then treated with trypsin (10%, wt/wt) and endopeptidase LysC (10%, wt/wt) in Tris·HCl buffer (0.1 M; pH 8.2) containing 5% acetonitrile. The peptides obtained were separated on HPLC by use of a μBondapak C_{18} reversed-phase column (Waters) developed with a 0–80% acetonitrile gradient in 0.1% trifluoroacetic acid. Peptide sequencing was carried out at the Rockefeller University Peptide Sequencing Facility.

Isolation of the cDNA Clone for GSH Synthetase. A rat kidney cDNA λgt11 expression library (Clontech) was immunoscreened as described (19) using the antiserum against rat kidney GSH synthetase preabsorbed with *E. coli* total protein. An overnight culture of *E. coli* Y1090r$^-$ was incubated with 10^6 plaque-forming units (pfu) of bacteriophage λgt11 at 37°C for 15 min. A suspension of the infected bacteria was poured onto 20 LB agar plates (150 × 35 mm) and incubated at 42°C for 3 h. The plates were covered with isopropyl β-D-thiogalactopyranoside-treated nitrocellulose membranes and incubated at 37°C for an additional 4 h. The filters were carefully peeled off the plates and rinsed with TNT buffer (10 mM Tris·HCl, pH 8.0/150 mM NaCl/0.05% Tween 20). The plates were covered with a second set of nitrocellulose filters and incubated at 37°C for 6 h.

The two sets of nitrocellulose filters were treated with blocking buffer (TNT buffer containing 5% nonfat dry milk) for 1 h. The filters were treated with TNT buffer containing diluted (1:200) antibody to rat kidney GSH synthetase for an additional 4 h. After washing three times with TNT buffer, the filters were then incubated with diluted peroxidase-linked goat anti-rabbit IgG antibody (1:5000) for 1 h. After washing five times, the antibody was visualized by treating the filters with Tris·HCl buffer (50 mM; pH 7.5) containing 0.018% H_2O_2 and 0.06% 3,3′-diaminobenzidine for 5 min. The plaques that appeared positive on both sets of the filters were picked, grown, and rescreened with the same antibody. The positive clone was confirmed by Western blot analysis using the total protein obtained from the *E. coli* infected with the phage in the positive plaque.

Purification of Recombinant λ DNA. The purification was performed as described (20). Recombinant λ phage particles (1×10^6 pfu) obtained from the positive clone were incubated with *E. coli* Y1090r$^-$ (1×10^8 cells) at 37°C for 20 min. The infected cells were inoculated into 100 ml of NZCYM medium until the cells lysed. After removal of cellular debris by centrifugation (8000 × *g*; 5 min), the λ phage was sedimented (25,000 rpm, SW28 rotor; 2 h). An aqueous solution of the phage was then treated with phenol and saturated with Tris buffer (pH 8), and the aqueous layer was treated with phenol/chloroform (1:1); the remaining aqueous layer was processed as described (19) to obtain the DNA. The DNA was precipitated by adding ethanol and dissolved in 50 μl of Tris·HCl (10 mM; pH 7.6) buffer containing 1 mM EDTA.

DNA Sequence Analysis. The insert cDNA in the λ DNA was excised by treatment with restriction enzyme *Bsi*WI and isolated by agarose gel electrophoresis. The cDNA was then blunt-ended with *E. coli* DNA polymerase I Klenow fragment and subcloned into the *Sma* I site of pBluescript KS(+). The nucleotide sequence was determined by the dideoxynucleotide chain-termination method (21) with Sequenase (United States Biochemical) according to the manufacturer's instructions. Sequence analysis was carried out with the PC/Gene software at the Rockefeller University Computer Service.

Northern Blot Analysis. A charged nylon membrane containing mRNA (2 μg each) from various rat tissues (Clontech) was used. The membrane was incubated with the prehybridization buffer (5× SSPE containing 10× Denhardt's solution, 50% formamide, 2% SDS, and 100 μg of denatured salmon sperm DNA) (19) at 42°C for 18 h with the *Sma* I fragment (682 bp) of the synthetase cDNA labeled with ^{32}P by nick-translation (≈2×10^8 cpm/μg). The membrane was washed twice with 2× SSPE containing 0.05% SDS at room temperature and twice for 40 min each in 0.1× SSPE containing 0.1% SDS at 50°C. Autoradiography was performed at −70°C for 4 days.

RESULTS

Characterization of the Peptides Obtained from Rat Kidney GSH Synthetase. Tryptic peptides of rat kidney GSH synthetase were separated on a C_{18} reversed-phase column. Two apparently homogeneous peptides were obtained and their sequences were determined by the automated Edman degradation method (peptides 1 and 2; Fig. 1). To obtain larger peptides, the protein was treated with endopeptidase LysC. Three peptides were obtained and sequenced (peptides 3–5; Fig. 1).

Peptide 1	A S Y I L M E K
Peptide 2	S C A A K
Peptide 3	Q L E E L A Q Q A I D R A L A E
Peptide 4	E R N I F D Q R A I E N E L L D R K
Peptide 5	A I E H A D G G V

FIG. 1. Amino acid sequence of peptides obtained from isolated rat kidney GSH synthetase. Peptides were isolated and sequenced as described. Peptides 1 and 2 were obtained after trypsin cleavage. Peptides 3–5 were obtained after endopeptidase LysC digestion.

Isolation of the cDNA Clone Coding for Rat Kidney GSH Synthetase. A rat kidney cDNA λgt11 expression library, which contains cDNA inserts in the phage *Eco*RI site, was screened with the antibody against rat kidney GSH synthetase. One clone was obtained from ≈10^6 phages. The clone was confirmed to be positive by the finding that the expressed fusion protein reacted with the antibody as determined by Western blot analysis (data not shown). An attempt to excise the cDNA insert by treating the λ DNA with *Eco*RI failed, indicating that the site was lost during construction of the cDNA library. The DNA was subsequently treated with *Bsi*WI, which cut the λ DNA at sites that were ≈200 bp away from the ends of the cDNA insert, and the insert DNA of 2.3 kb was separated by agarose gel electrophoresis. The cDNA was blunt-ended and subcloned into the *Sma* I site of pBluescript KS(+) for sequence analysis.

Nucleotide Sequence of the cDNA for Rat Kidney GSH Synthetase. The cDNA sequence corresponding to rat kidney GSH synthetase mRNA is presented in Fig. 2. The entire positive strand was sequenced at least twice from different overlapping sets using internal primers. The sequence was confirmed completely by sequencing the complementary strand. The sequence contains 1905 nucleotides and an open reading frame of 1422 nucleotides that codes for 474 amino acid residues. The cDNA also has a 3′ nontranslated region of 439 nucleotides including a poly(A) tail. The first ATG at position 1 is thought to be the translation start codon. The open reading frame starting with this ATG codon encodes a protein of M_r 52,344, which is in close agreement with the value M_r 55,000 estimated by SDS gel electrophoresis of the purified rat kidney enzyme. The open reading frame sequence ends with the termination codon TGA at position 1423.

Analysis of the Predicted Amino Acid Sequence of Rat Kidney GSH Synthetase. The deduced amino acid sequence contains all the five independently determined peptide sequences: residues 14–29, 214–231, 289–293, 393–400, and 453–461 (Figs. 1 and 2). Thus, ≈12% of the amino acid residues of the enzyme were independently determined by the Edman procedure. Two potential N-linked glycosylation sites (residues 124 and 171) and one possible O-linked *N*-acetylglucosamine addition site (residue 389) are found in the sequence. This enzyme has previously been shown to contain a small amount of carbohydrate. Hydropathy analysis (22) (data not shown) revealed a pattern typical of a water-soluble protein. The calculated amino acid composition is in good agreement with the amino acid analyses of the isolated rat kidney GSH synthetase (16) (data not shown).

Tissue mRNA Expression. Northern blot analysis of the rat tissue mRNA using a ^{32}P-labeled GSH synthetase probe revealed a 2.1-kb mRNA band. Rat kidney gave the strongest signal among the tissues examined (heart, brain, spleen, lung, liver, skeletal muscle, kidney, testis) consistent with the finding that kidney exhibits the highest GSH synthetase activity among the various tissues. The intensity of the band found with liver was ≈5% that found in kidney.

Expression of the cDNA in *E. coli*. The cDNA encoding rat kidney GSH synthetase was inserted into expression vector pT7-7 (a gift of Stanley Tabor, Harvard University) and transformed into *E. coli* BL21(DE3). The recombinant GSH synthetase was expressed as described by Huang *et al.* (9). Greater than 8000 units of the recombinant GSH synthetase (above the control) was obtained from 1 liter of *E. coli* culture. Since *E. coli* is not expected to glycosylate proteins, it appears that the carbohydrate moieties of rat kidney GSH synthetase are not required for the enzyme activity.

DISCUSSION

This study provides the amino acid sequence of GSH synthetase from a mammalian source. The cDNA sequence contains 1905 nucleotides with an open reading frame of 1422 nucleotides coding for 474 amino acid residues. The deduced amino acid sequence leads to a M_r of 52,344, which is not far from previous estimates of 55,000 based on SDS gel electrophoresis of isolated rat kidney GSH synthetase. The enzyme was found to have a M_r of 118,000 as determined by gel filtration chromatography, suggesting that the rat kidney enzyme is composed of two apparently identical subunits. All of the five independently determined peptide sequences were found in various sections of the cDNA sequence. The data support the conclusion that the two subunits are identical. The GSH synthetases isolated from *Aspergillus niger* (M_r, 110,000) (23) and bovine erythrocytes (M_r, 121,000) (24) also contain two apparently identical subunits.

The GSH synthetase of *E. coli* was isolated by Gushima *et al.* (25, 26), who found that the purified enzyme had a M_r of 152,000 and was composed of four apparently identical subunits of M_r 38,000; the enzyme was cloned and sequenced. The enzymes purified from bakers' yeast (27) and fission yeast (28) have a M_r of ≈120,000 each. The fission yeast enzyme is reported to be a heterotetramer composed of two subunits of M_r 33,000 and 26,000 (28). The DNA that codes for the heavy subunit was isolated and partially sequenced. When the heavy subunit DNA was introduced into the yeast, both the heavy subunit and the light subunit were overexpressed. The authors concluded that the enzyme is composed of two kinds of subunits and that it has an A_2B_2 structure. The gene for the large subunit of *Schizosaccharomyces pombe* was cloned from a *S. pombe* genomic DNA library by complementation of cadmium ion hypersensitivity of a GSH synthetase-deficient *S. pombe* mutant (29). Cadmium ions (and certain other metal ions) induce formation of phytochelatins (30) [cadystins (29)], which have the general structure (γ-Glu-Cys)$_n$-Gly. The mechanisms involved in the formation of these peptides remain to be determined, and the possibility that GSH synthetase activity is involved has been suggested (29). Putative cDNA for frog GSH synthetase was isolated by using degenerated oligonucleotides derived arbitrarily from the deduced fission yeast amino acid sequence (31). The proposed subunit structure of the frog enzyme needs to be substantiated since the enzyme has not yet been isolated.

Computer-aided comparisons of the amino acid sequence of rat kidney GSH synthetase with the sequences of other GSH synthetases in the literature were carried out. Comparisons of the rat enzyme sequence with that of *E. coli* (25, 26) showed no significant similarity. About 30% similarity was found with the yeast enzyme (28) and ≈65% similarity was found with the sequence reported for the frog (31).

It was initially believed that glycoproteins are synthesized in the endoplasmic reticulum and Golgi apparatus and delivered to the cell membrane. However, recent evidence indicates that glycoproteins also exist in the cytoplasm (32, 33). The majority of the cytoplasmic glycoproteins are modified by O-linked *N*-acetylglucosamine, but other types of modification are also present. Isolated rat kidney GSH synthetase is known to contain a small amount (2%) of carbohydrate (16). There are two asparagine residues (residues 124 and 171) in the deduced amino acid sequence that fit the requirement for N-linked protein glycosylation: Asn-Xaa-Ser/Thr (Xaa, any amino acid residue except proline), and one serine residue (residue 389) agrees with the general pattern for an O-linked *N*-acetylglucosamine addition site (Asp)-Ser/Thr-Xaa$_n$-Pro. Further study on the significance of the glycosylation of this enzyme is needed.

Availability of the amino acid sequence of rat kidney GSH synthetase will facilitate determination of the amino acid sequence of the human enzyme. This may be expected to be valuable for understanding the biochemical defects associated with human GSH synthetase deficiency and for development

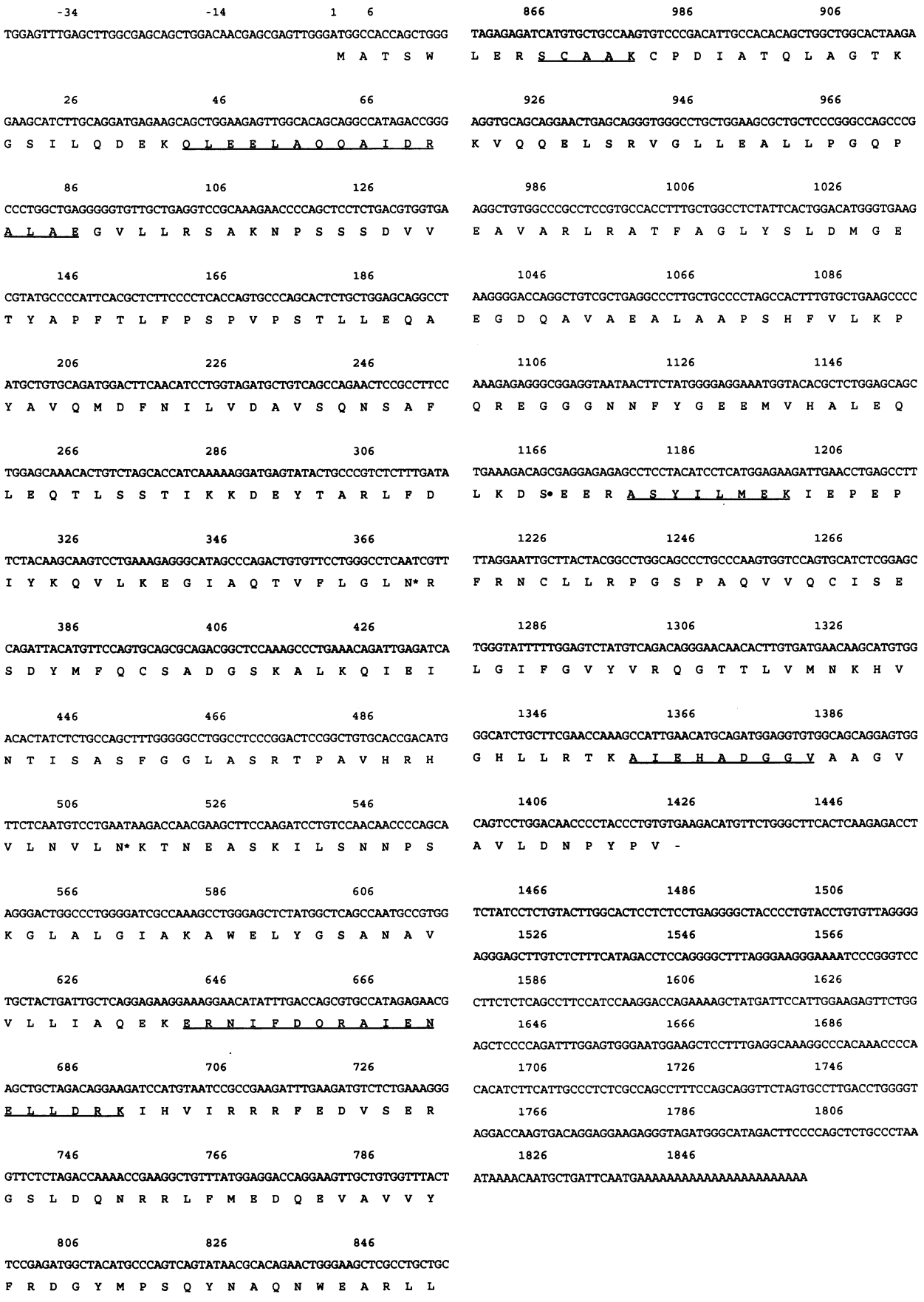

FIG. 2. cDNA sequence coding for rat kidney GSH synthetase. cDNA contains 1905 nucleotides. Open reading frame begins at nucleotide 1 and ends at nucleotide 1423. Deduced amino acid sequence is shown under the cDNA sequence. Independently determined peptide sequences are underlined. *, Potential N-linked carbohydrate binding site; ●, possible O-linked carbohydrate binding site.

of improved methods for prenatal diagnosis of the human diseases associated with defects in GSH synthetase.

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