

DESCRIPTION

ENGINEERED PRIMATE CYSTINE/CYSTEINE DEGRADING ENZYMES FOR THERAPEUTIC USES

REFERENCE TO RELATED APPLICATIONS

5 **[0001]** The present application claims the priority benefit of United States provisional application number 62/751,197, filed October 26, 2018, the entire contents of which is incorporated herein by reference.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

10 **[0002]** This invention was made with government support under Grant No. R01 CA189623 awarded by the National Institutes of Health. The government has certain rights in the invention.

REFERENCE TO A SEQUENCE LISTING

15 **[0003]** The instant application contains a Sequence Listing, which has been submitted in ASCII format via EFS-Web and is hereby incorporated by reference in its entirety. Said ASCII copy, created on October 22, 2019, is named UTFBP1212WO_ST25.txt and is 365 kilobytes in size.

PARTIES TO JOINT RESEARCH AGREEMENT

20 **[0004]** The inventions disclosed and claimed herein were developed within the scope of a Joint Research Agreement between Aeglea BioTherapeutics, Inc. and The Board of Regents of The University of Texas System.

BACKGROUND

1. Field

25 **[0005]** Disclosed are recombinantly engineered primate enzyme variants having high cysteine/cysteine degrading activity suitable for human therapy. Compositions and methods for the treatment of cystinuria with enzymes that deplete both L-cystine and L-cysteine are also provided.

2. Description of Related Art

[0006] Cystinuria is a hereditary disorder caused by mutations in the *SLC3A1* and *SLC7A9* genes encoding the kidney proximal tubule's cystine and dibasic amino acid transporter that leads to abnormal excretion of cystine (the disulfide form of the amino acid cysteine) and the formation of cystine crystals/stones in the urinary tract. There are few therapeutics available to patients suffering from the hereditary disorder cystinuria wherein a defective kidney transporter is unable to re-uptake cystine during renal filtration. Cystine, the disulfide form of the amino acid L-cysteine, is highly insoluble and in cystinuria patients reaches high concentrations in the urinary tract resulting in the formation of cystine crystals and stones. Existing therapies that reduce circulating cystine levels partially prevent urinary tract stone formation but have significant adverse effects that limit their use. Therapies are needed to reduce and prevent cystine stone formation in the kidney and bladder.

SUMMARY

[0007] In one aspect, the present disclosure refers to an isolated, modified primate cystathionine- γ -lyase (CGL) enzyme comprising the following substitutions relative to a native human CGL amino acid sequence (SEQ ID NO: 1), wherein the modified enzyme has both cystinase and cysteinase activity, said substitutions being: alanine at position 193, glycine at position 311, valine at position 339, and serine at position 353. The present disclosure describes the engineering of primate cystathionine-gamma-lyase ("CGL") enzymes such that both L-cystine and L-cysteine (referred to herein as "L-cyst(e)ine") can be efficiently degraded from serum, and providing the modified CGL enzymes in a formulation suitable for human therapy. To develop an enzyme displaying low K_M and high catalytic activity, k_{cat} , as compared to the native enzyme, the native enzyme was engineered by modifying selected amino acids, which modifications result in an enzyme having dramatically improved enzymatic properties. As such, modified CGL enzymes, as described herein, overcome a major deficiency in the art by providing novel enzymes that comprise human or primate polypeptide sequences having improved L-cyst(e)ine-degrading catalytic activity. As this enzyme is comprised of a human sequence, it is not likely to induce adverse immunological responses. As such, these modified enzymes may be suitable for human therapy and have low immunogenicity.

[0008] Methods are disclosed of utilizing an engineered human cystathionine-gamma-lyase (CGL) enzyme that efficiently converts cystine to cysteine-persulfide, which subsequently decays to free cysteine and H_2S , such that it is a suitable therapy for treating cystinuria patients by preventing cystine accumulation and formation of stones in the kidney and urinary tract. In addition, the engineered human CGL enzyme can convert cysteine to pyruvate, ammonia, and hydrogen sulfide, in effect reducing the amount of cysteine that can oxidize to form cystine. As cystine is a non-essential amino acid, which is normally

produced by most cells, no toxicities have been found to be induced by long-term cystine depletion in animal models. The ability of a cystine-degrading therapeutic to non-toxically ablate the total levels of circulating cystine indicate that it would be a superior therapeutic regimen for preventing cystine stone formation than existing therapeutic regimens.

5 **[0009]** Disclosed herein are modified CGL enzymes, having L-cyst(e)ine degrading activity, that are derived from primate CGL enzymes. A modified CGL enzyme may be derived from a human CGL enzyme (SEQ ID NO: 1), a *Pongo abelii* CGL enzyme (Genbank ID: NP_001124635.1; SEQ ID NO: 2), a *Macaca fascicularis* CGL enzyme (Genbank ID: AAW71993.1; SEQ ID NO: 3), a *Pan troglodytes* CGL enzyme (Genbank ID: XP_513486.2; SEQ ID NO: 4), or a *Pan paniscus* CGL enzyme (Genbank ID: XP_003830652.1; SEQ ID NO: 5). The native CGL enzyme may be modified by one or more other modifications, such as chemical modifications, substitutions, insertions, deletions, and/or truncations.

15 **[0010]** A modified CGL enzyme may be derived from a native, primate CGL enzyme by modifying by one, two, three, four or more substitutions at amino acid position(s) 51, 55, 59, 91, 163, 189, 193, 200, 234, 311, 336, 339, and/or 353 of SEQ ID NOs: 1-5. In these examples, the first methionine of each sequence corresponds to amino acid position 1, and each amino acid is numbered sequentially therefrom. The substitutions at amino acid positions 51 may be tryptophan (W), 55 may be glutamic acid (E), 59 may be threonine (T) or isoleucine (I), 91 may be methionine (M) or serine (S), 163 may be arginine (R), 189 may be serine (S), 193 may be glycine (G) or alanine (A), 200 may be proline (P) or histidine (H), 234 may be lysine (K), 311 may be glycine (G), 336 may be aspartic acid (D), 339 may be valine (V), and/or 353 may be serine (S).

25 **[0011]** A modified CGL enzyme may have an amino acid sequence according to SEQ ID NOs: 6-95. A modified CGL enzyme may be capable of degrading L-cyst(e)ine under physiological conditions. The modified CGL enzyme may have a catalytic efficiency for L-cyst(e)ine (k_{cat}/K_M) of at least or about 1000, 2000, 3000, 4000, 5000, 6000, 7000, 8000, 9000, 10^4 , 10^5 , 10^6 s⁻¹M⁻¹ or any range derivable therein. An exemplary CGL enzyme may have a catalytic efficiency of $>10^4$ s⁻¹M⁻¹ for L-cystine and $>10^3$ s⁻¹M⁻¹ for L-cysteine.

30 **[0012]** The substitutions may be a combination of P193A, T311G, E339V, and I353S of human CGL (for example, the modified polypeptide having the amino acid sequence of SEQ ID NO: 6 or a fragment or homolog thereof). For example, an equivalent substitution of

I353 in SEQ ID NO: 1 for SEQ ID NO: 2 would modify a valine and not isoleucine as in SEQ ID NO: 1. The substitutions may be a combination of A200P, T311G, E339V, and I353S of human CGL (for example, the modified polypeptide having the amino acid sequence of SEQ ID NO: 7 or a fragment or homolog thereof). The substitutions may be a combination of P193A, A200P, T311G, E339V, and I353S of human CGL (for example, the modified polypeptide having the amino acid sequence of SEQ ID NO: 8 or a fragment or homolog thereof). The substitutions may be a combination of H55E, E59T, L91M, N234K, T336D, and E339V of human CGL (for example, the modified polypeptide having the amino acid sequence of SEQ ID NO: 9 or a fragment or homolog thereof). For example, an equivalent substitution of E59 in SEQ ID NO: 1 for SEQ ID NO: 2 would modify a valine and not glutamine acid as in SEQ ID NO: 1. The substitutions may be a combination of H55E, E59T, L91S, N234K, T336D, and E339V of human CGL (for example, the modified polypeptide having the amino acid sequence of SEQ ID NO: 10 or a fragment or homolog thereof). The substitutions may be a combination of T189S, P193G, T311G, E339V, and I353S of human CGL (for example, the modified polypeptide having the amino acid sequence of SEQ ID NO: 11 or a fragment or homolog thereof). The substitutions may be a combination of T163R, T311G, E339V, and I353S of human CGL (for example, the modified polypeptide having the amino acid sequence of SEQ ID NO: 12 or a fragment or homolog thereof). For example, an equivalent substitution of T163 in SEQ ID NO: 1 for SEQ ID NO: 3 would modify a valine and not threonine as in SEQ ID NO: 1. The substitutions may be a combination of A51W, H55E, E59T, T336D, and E339V of human CGL (for example, the modified polypeptide having the amino acid sequence of SEQ ID NO: 13 or a fragment or homolog thereof). The substitutions may be a combination of H55E, P193A, T311G, T336D, E339V, and I353S of human CGL (for example, the modified polypeptide having the amino acid sequence of SEQ ID NO: 14 or a fragment or homolog thereof). The substitutions may be a combination of A200H, T311G, E339V, and I353S of human CGL (for example, the modified polypeptide having the amino acid sequence of SEQ ID NO: 15 or a fragment or homolog thereof). The substitutions may be a combination of T311G, E339V, and I353S of human CGL (for example, the modified polypeptide having the amino acid sequence of SEQ ID NO: 16 or a fragment or homolog thereof). The substitutions may be a combination of E59I, E339V, and I353S of human CGL (for example, the modified polypeptide having the amino acid sequence of SEQ ID NO: 17 or a fragment or homolog thereof). The substitutions may be a combination of L91M and E339V of human CGL (for example, the modified polypeptide having the amino acid sequence of SEQ ID NO:

18 or a fragment or homolog thereof). The substitutions may be a combination of E339V and I353S of human CGL (for example, the modified polypeptide having the amino acid sequence of SEQ ID NO: 19 or a fragment or homolog thereof). The substitution may be E339V of human CGL (for example, the modified polypeptide having the amino acid
 5 sequence of SEQ ID NO: 20 or a fragment or homolog thereof).

[0013] The modified polypeptide may be a *Pongo abelii* CGL-P193A-T311G-E339V-V353S mutant (SEQ ID NO: 24), *Pongo abelii* CGL-A200P-T311G-E339V-V353S mutant (SEQ ID NO: 25), *Pongo abelii* CGL-P193A-A200P-T311G-E339V-V353S mutant (SEQ ID NO: 26), *Pongo abelii* CGL-H55E-V59T-L91M-N234K-T336D-E339V mutant
 10 (SEQ ID NO: 27), *Pongo abelii* CGL-H55E-V59T-L91S-N234K-T336D-E339V mutant (SEQ ID NO: 28), *Pongo abelii* CGL-T189S-P193G-T311G-E339V-V353S mutant (SEQ ID NO: 29), *Pongo abelii* CGL-T163R-T311G-E339V-V353S mutant (SEQ ID NO: 30), *Pongo abelii* CGL-A51W-H55E-V59T-T336D-E339V mutant (SEQ ID NO: 31), *Pongo abelii* CGL-H55E-P193A-T311G-T336D-E339V-V353S mutant (SEQ ID NO: 32), *Pongo abelii*
 15 CGL-A200H-T311G-E339V-V353S mutant (SEQ ID NO: 33), *Pongo abelii* CGL-T311G-E339V-V353S mutant (SEQ ID NO: 34), *Pongo abelii* CGL-V59I-E339V-V353S mutant (SEQ ID NO: 35), *Pongo abelii* CGL-L91M-E339V mutant (SEQ ID NO: 36), *Pongo abelii* CGL-E339V-V353S mutant (SEQ ID NO: 37), or *Pongo abelii* CGL-E339V mutant (SEQ ID NO: 38).

20 [0014] The modified polypeptide may be a *Macaca fascicularis* CGL-P193A-T311G-E339V-I353S mutant (SEQ ID NO: 42), *Macaca fascicularis* CGL-A200P-T311G-E339V-I353S mutant (SEQ ID NO: 43), *Macaca fascicularis* CGL-P193A-A200P-T311G-E339V-I353S mutant (SEQ ID NO: 44), *Macaca fascicularis* CGL-H55E-E59T-L91M-N234K-T336D-E339V mutant (SEQ ID NO: 45), *Macaca fascicularis* CGL-H55E-E59T-L91S-
 25 N234K-T336D-E339V mutant (SEQ ID NO: 46), *Macaca fascicularis* CGL-T189S-P193G-T311G-E339V-I353S mutant (SEQ ID NO: 47), *Macaca fascicularis* CGL-V163R-T311G-E339V-I353S mutant (SEQ ID NO: 48), *Macaca fascicularis* CGL-A51W-H55E-E59T-T336D-E339V mutant (SEQ ID NO: 49), *Macaca fascicularis* CGL-H55E-P193A-T311G-T336D-E339V-I353S mutant (SEQ ID NO: 50), *Macaca fascicularis* CGL-A200H-T311G-
 30 E339V-I353S mutant (SEQ ID NO: 51), *Macaca fascicularis* CGL-T311G-E339V-I353S mutant (SEQ ID NO: 52), *Macaca fascicularis* CGL-E59I-E339V-I353S mutant (SEQ ID NO: 53), *Macaca fascicularis* CGL-L91M-E339V mutant (SEQ ID NO: 54), *Macaca*

fascicularis CGL-E339V-I353S mutant (SEQ ID NO: 55), or *Macaca fascicularis* CGL-E339V mutant (SEQ ID NO: 56).

[0015] The modified polypeptide may be a *Pan troglodytes* CGL-P193A-T311G-E339V-I353S mutant (SEQ ID NO: 60), *Pan troglodytes* CGL-A200P-T311G-E339V-I353S mutant (SEQ ID NO: 61), *Pan troglodytes* CGL-P193A-A200P-T311G-E339V-I353S mutant (SEQ ID NO: 62), *Pan troglodytes* CGL-H55E-E59T-L91M-N234K-T336D-E339V mutant (SEQ ID NO: 63), *Pan troglodytes* CGL-H55E-E59T-L91S-N234K-T336D-E339V mutant (SEQ ID NO: 64), *Pan troglodytes* CGL-T189S-P193G-T311G-E339V-I353S mutant (SEQ ID NO: 65), *Pan troglodytes* CGL-T163R-T311G-E339V-I353S mutant (SEQ ID NO: 66), *Pan troglodytes* CGL-A51W-H55E-E59T-T336D-E339V mutant (SEQ ID NO: 67), *Pan troglodytes* CGL-H55E-P193A-T311G-T336D-E339V-I353S mutant (SEQ ID NO: 68), *Pan troglodytes* CGL-A200H-T311G-E339V-I353S mutant (SEQ ID NO: 69), *Pan troglodytes* CGL-T311G-E339V-I353S mutant (SEQ ID NO: 70), *Pan troglodytes* CGL-E59I-E339V-I353S mutant (SEQ ID NO: 71), *Pan troglodytes* CGL-L91M-E339V mutant (SEQ ID NO: 72), *Pan troglodytes* CGL-E339V-I353S mutant (SEQ ID NO: 73), or *Pan troglodytes* CGL-E339V mutant (SEQ ID NO: 74).

[0016] The modified polypeptide may be a *Pan paniscus* CGL-P193A-T311G-E339V-I353S mutant (SEQ ID NO: 78), *Pan paniscus* CGL-A200P-T311G-E339V-I353S mutant (SEQ ID NO: 79), *Pan paniscus* CGL-P193A-A200P-T311G-E339V-I353S mutant (SEQ ID NO: 80), *Pan paniscus* CGL-H55E-E59T-L91M-N234K-T336D-E339V mutant (SEQ ID NO: 81), *Pan paniscus* CGL-H55E-E59T-L91S-N234K-T336D-E339V mutant (SEQ ID NO: 82), *Pan paniscus* CGL-T189S-P193G-T311G-E339V-I353S mutant (SEQ ID NO: 83), *Pan paniscus* CGL-T163R-T311G-E339V-I353S mutant (SEQ ID NO: 84), *Pan paniscus* CGL-A51W-H55E-E59T-T336D-E339V mutant (SEQ ID NO: 85), *Pan paniscus* CGL-H55E-P193A-T311G-T336D-E339V-I353S mutant (SEQ ID NO: 86), *Pan paniscus* CGL-A200H-T311G-E339V-I353S mutant (SEQ ID NO: 87), *Pan paniscus* CGL-T311G-E339V-I353S mutant (SEQ ID NO: 88), *Pan paniscus* CGL-E59I-E339V-I353S mutant (SEQ ID NO: 89), *Pan paniscus* CGL-L91M-E339V mutant (SEQ ID NO: 90), *Pan paniscus* CGL-E339V-I353S mutant (SEQ ID NO: 91), or *Pan paniscus* CGL-E339V mutant (SEQ ID NO: 92).

[0017] A modified CGL enzyme as discussed herein may be characterized as having a certain percentage of identity as compared to an unmodified CGL enzyme (*e.g.*, a native CGL

enzyme). For example, the unmodified CGL enzyme may be a native primate cystathionase (*i.e.*, cystathionine- γ -lyase). The percentage identity may be at least 90%, 95%, 96%, 97%, 98%, 99% or 100% (or any range derivable therein) between the unmodified portions of a modified CGL enzyme (*i.e.*, the sequence of the modified CGL enzyme excluding any substitutions at amino acid positions 51, 55, 59, 91, 163, 189, 193, 200, 234, 311, 336, 339, and/or 353 of SEQ ID NO: 1-5, see FIG. 1) and the native CGL enzyme. It is also contemplated that the percent identity discussed above may relate to a particular modified region of an enzyme as compared to an unmodified region of the corresponding native enzyme. For instance, a modified CGL enzyme may contain a modified or mutant substrate recognition site that can be characterized based on the identity of the amino acid sequence of the modified or mutant substrate recognition site to that of an unmodified or native CGL enzyme from the same species or across species. For example, a modified human CGL enzyme characterized as having at least 90% identity to an unmodified human CGL enzyme means that at least 90% of the amino acids in the modified human CGL enzyme are identical to the amino acids in the unmodified human CGL enzyme.

[0018] A modified CGL enzyme can be linked to a heterologous peptide sequence or polysaccharide. For example, a modified CGL enzyme may be linked to the heterologous peptide sequence as a fusion protein. The modified CGL enzyme may be linked to amino acid sequences, such as an IgG Fc, albumin, an albumin binding peptide, or an XTEN polypeptide for increasing the *in vivo* half-life. The modified CGL enzyme may be linked to a polysialic acid polymer.

[0019] To increase serum persistence, the modified CGL enzyme may be linked to one or more polyether molecules. The polyether may be polyethylene glycol (PEG). The modified CGL enzyme may be linked to PEG *via* specific amino acid residues, such as lysine or cysteine. For therapeutic administration, as such the modified CGL enzyme may be dispersed in a pharmaceutically acceptable carrier.

[0020] A nucleic acid encoding a modified CGL enzyme is contemplated. The nucleic acid can be codon optimized for expression in bacteria, such as for *E. coli*. Nucleic acids that are codon optimized for the expression of the modified CGL enzymes provided in SEQ ID NOs: 6-20 in *E. coli* are provided in SEQ ID NOs: 96-110, respectively. Alternatively, the nucleic acid can be codon optimized for expression in fungus (*e.g.*, yeast), insects, or mammals. Further contemplated are vectors, such as expression vectors,

containing such nucleic acids. A nucleic acid encoding the modified CGL enzyme can be operably linked to a promoter, including but not limited to heterologous promoters. A modified CGL enzyme may be delivered to a target cell by a vector (*e.g.*, a gene therapy vector). Such vectors may have been modified by recombinant DNA technology to enable the expression of the modified CGL-encoding nucleic acid in the target cell. These vectors may be derived from vectors of non-viral (*e.g.*, plasmids) or viral (*e.g.*, adenovirus, adeno-associated virus, retrovirus, lentivirus, herpes virus, or vaccinia virus) origin. Non-viral vectors may be complexed with agents to facilitate the entry of the DNA across the cellular membrane. Examples of such non-viral vector complexes include the formulation with polycationic agents, which facilitate the condensation of the DNA, and lipid-based delivery systems. Lipid-based delivery systems include liposome-based delivery of nucleic acids.

[0021] Host cells comprising such vectors are provided. The host cells may be bacteria (*e.g.*, *E. coli*), fungal cells (*e.g.*, yeast), insect cells, or mammalian cells.

[0022] A vector can be introduced into host cells for expressing the modified CGL enzyme. The modified CGL enzymes may be expressed in any suitable manner. The modified CGL enzymes may be expressed in a host cell such that the protein is glycosylated. Alternatively, the modified CGL enzymes may be expressed in a host cell such that the protein is aglycosylated.

[0023] Therapeutic formulations containing the modified CGL enzyme and a pharmaceutically acceptable carrier are provided. Therapeutic formulations can be administered intravenously, intradermally, intraarterially, intraperitoneally, intramuscularly, subcutaneously, by infusion, by continuous infusion, *via* a catheter, in lipid compositions (*e.g.*, liposomes).

[0024] In one aspect, the present disclosure refers to a use of a formulation as disclosed herein in the manufacture of a medicament for treating a subject having or at risk of developing cystinuria. Methods are disclosed for treating a subject having or being at risk of developing cystinuria, the methods comprising administering to the subject a therapeutically effective amount of a formulation comprising an isolated, modified primate CGL enzyme having at least one substitution relative to a native primate CGL amino acid sequence (see SEQ ID NOs: 1-5) or a nucleic acid comprising a nucleotide sequence encoding the isolated, modified primate CGL enzyme. The isolated, modified primate CGL enzyme may have a sequence according to any one of SEQ ID NOs: 6-95. The methods may reduce the level of cysteine and/or cystine in the subject's plasma, serum, and/or urine.

[0025] The subject may be any animal, such as a mouse. For example, the subject may be a mammal, a rodent, a primate, or a human patient. The subject or patient may be maintained on a L-cyst(e)ine-restricted diet, a methionine-restricted diet or a normal diet in combination with being treated with the described compositions.

5 [0026] The subject may have previously been treated for cystinuria and the CGL enzyme is administered to treat or moderate the recurrence of cystinuria or ameliorate one or more conditions and/or symptoms associated with cystinuria. The methods may also comprise administering at least a second therapy to the subject, such as a second cystinuria therapy, such as modifications of diet, nutritional therapy, and shock wave therapy. The methods may reduce the
10 volume of cystine stones or the number of cystine stones in the subject's urinary tract, kidney, and/or bladder. The methods may prevent the formation of cystine stones in the subject's urinary tract, kidney, and/or bladder. The methods may reduce the number of cystine crystals in the subject's urine. The methods may prevent the formation of cystine crystals in the subject's urine.

[0027] As such, methods are provided for preventing the formation of cystine stones
15 in patients having cystinuria, the methods comprising administering to the subject a therapeutically effective amount of a formulation comprising an isolated, modified primate CGL enzyme having at least one substitution relative to a native primate CGL amino acid sequence (see SEQ ID NOs: 1-5) or a nucleic acid comprising a nucleotide sequence encoding the isolated, modified primate CGL enzyme. The isolated, modified primate CGL
20 enzyme may have a sequence according to any one of SEQ ID NOs: 6-95. The methods may prevent the formation of cystine stones in the patient's kidney and/or bladder.

[0028] A composition comprising a modified CGL enzyme or a nucleic acid encoding a modified CGL enzyme is provided for use in the treatment of cystinuria in a subject. The use of an isolated or modified CGL enzyme, a nucleic acid encoding a modified CGL
25 enzyme, or a composition comprising said modified CGL enzyme in the manufacture of a medicament for therapeutic application to a cystinuria patient is provided. The modified CGL enzyme may be any modified CGL enzyme disclosed herein.

[0029] Also provided herein are:

1. In one aspect, the present disclosure refers to an isolated, modified primate
30 cystathionine- γ -lyase (CGL) enzyme comprising at least the following substitutions relative to a native human CGL amino acid sequence (SEQ

ID NO: 1), wherein the modified enzyme has both cystinase and cysteinase activity, said substitutions being selected from the group consisting of:

- (a) alanine at position 193, glycine at position 311, valine at position 339, and serine at position 353;
- 5 (b) proline at position 200, glycine at position 311, valine at position 339, and serine at position 353;
- (c) alanine at position 193, proline at position 200, glycine at position 311, valine at position 339, and serine at position 353;
- 10 (d) glutamic acid at position 55, threonine at position 59, methionine at position 91, lysine at position 234, aspartic acid at position 336, and valine at position 339;
- (e) glutamic acid at position 55, threonine at position 59, serine at position 91, lysine at position 234, aspartic acid at position 336, and valine at position 339;
- 15 (f) serine at position 189, glycine at position 193, glycine at position 311, valine at position 339, and serine at position 353;
- (g) arginine at position 163, glycine at position 311, valine at position 339, and serine at position 353;
- (h) tryptophan at position 51, glutamic acid at position 55, threonine at position 59, aspartic acid at position 336, and valine at position 339;
- 20 (i) glutamic acid at position 55, alanine at position 193, glycine at position 311, aspartic acid at position 336, valine at position 339, and serine at position 353;
- (j) histidine at position 200, glycine at position 311, valine at position 339, and serine at position 353;
- (k) glycine at position 311, valine at position 339, and serine at position 353;
- 25 (l) isoleucine at position 59, valine at position 339, and serine at position 353;
- (m) methionine at position 91 and valine at position 339; and
- (n) valine at position 339 and serine at position 353.

2. The isolated, modified CGL enzyme as disclosed herein, wherein the modified CGL enzyme is a modified *Pongo abelii* CGL enzyme.

30 3. The isolated, modified CGL enzyme as disclosed herein, wherein the modified *Pongo abelii* CGL enzyme comprises substitutions selected from the group consisting of:

- (a) P193A, T311G, E339V, and V353S;

- (b) A200P, T311G, E339V, and V353S;
 - (c) P193A, A200P, T311G, E339V, and V353S;
 - (d) H55E, V59T, L91M, N234K, T336D, and E339V;
 - (e) H55E, V59T, L91S, N234K, T336D, and E339V;
 - 5 (f) T189S, P193G, T311G, E339V, and V353S;
 - (g) T163R, T311G, E339V, and V353S;
 - (h) A51W, H55E, V59T, T336D, and E339V;
 - (i) H55E, P193A, T311G, T336D, E339V, and V353S;
 - (j) A200H, T311G, E339V, and V353S;
 - 10 (k) T311G, E339V, and V353S;
 - (l) V59I, E339V, and V353S;
 - (m) L91M and E339V; and
 - (n) E339V and V353S.
4. The isolated, modified CGL enzyme as disclosed herein, wherein the modified CGL
- 15 enzyme is a modified human CGL enzyme, a modified *Pan troglodytes* CGL enzyme, or a modified *Pan paniscus* CGL enzyme.
5. The isolated, modified CGL enzyme as disclosed herein, wherein the modified human CGL enzyme, the modified *Pan troglodytes* CGL enzyme, or the modified *Pan paniscus* CGL enzyme comprises substitutions selected from the group consisting of:
- 20 (a) P193A, T311G, E339V, and I353S;
 - (b) A200P, T311G, E339V, and I353S;
 - (c) P193A, A200P, T311G, E339V, and I353S;
 - (d) H55E, E59T, L91M, N234K, T336D, and E339V;
 - (e) H55E, E59T, L91S, N234K, T336D, and E339V;
 - 25 (f) T189S, P193G, T311G, E339V, and I353S;
 - (g) T163R, T311G, E339V, and I353S;
 - (h) A51W, H55E, E59T, T336D, and E339V;
 - (i) H55E, P193A, T311G, T336D, E339V, and I353S;
 - (j) A200H, T311G, E339V, and I353S;
 - 30 (k) T311G, E339V, and I353S;
 - (l) E59I, E339V, and I353S;
 - (m) L91M and E339V; and

(n) E339V and I353S.

6. The isolated, modified CGL enzyme as disclosed herein, wherein the modified CGL enzyme is a modified *Macaca fascicularis* CGL enzyme.

7. The isolated, modified CGL enzyme as disclosed herein, wherein the modified *Macaca fascicularis* CGL enzyme comprises substitutions selected from the group consisting of:

- (a) P193A, T311G, E339V, and I353S;
- (b) A200P, T311G, E339V, and I353S;
- (c) P193A, A200P, T311G, E339V, and I353S;
- (d) H55E, E59T, L91M, N234K, T336D, and E339V;
- (e) H55E, E59T, L91S, N234K, T336D, and E339V;
- (f) T189S, P193G, T311G, E339V, and I353S;
- (g) V163R, T311G, E339V, and I353S;
- (h) A51W, H55E, E59T, T336D, and E339V;
- (i) H55E, P193A, T311G, T336D, E339V, and I353S;
- (j) A200H, T311G, E339V, and I353S;
- (k) T311G, E339V, and I353S;
- (l) E59I, E339V, and I353S;
- (m) L91M and E339V; and
- (n) E339V and I353S.

8. The isolated, modified CGL enzyme as disclosed herein, further comprising a heterologous peptide segment or a polysaccharide.

9. The isolated, modified CGL enzyme as disclosed herein, wherein the heterologous peptide segment is an XTEN peptide, an IgG Fc, an albumin, or an albumin binding peptide.

10. The isolated, modified CGL enzyme as disclosed herein, wherein the polysaccharide comprises polysialic acid polymers.

11. The isolated, modified CGL enzyme of any one as disclosed herein, wherein the enzyme is coupled to polyethylene glycol (PEG).

12. The isolated, modified CGL enzyme as disclosed herein, wherein the enzyme is coupled to the PEG via one or more lysine residues.

13. In another aspect, the present disclosure refers to a nucleic acid comprising a nucleotide sequence encoding the enzyme as disclosed herein.
14. The nucleic acid as disclosed herein, wherein the nucleic acid is codon optimized for expression in bacteria, fungus, insects, or mammals.
- 5 15. The nucleic acid as disclosed herein, wherein the bacteria are *E. coli*.
16. The nucleic acid as disclosed herein, wherein the nucleic acid comprises a sequence according to one of SEQ ID NOs: 81-95.
17. In yet another aspect, the present disclosure refers to an expression vector comprising the nucleic acid as disclosed herein.
- 10 18. In another aspect, the present disclosure refers to a host cell comprising the nucleic acid as disclosed herein.
19. The host cell as disclosed herein, wherein the host cell is a bacterial cell, a fungal cell, an insect cell, or a mammalian cell.
20. In another aspect, the present disclosure refers to a therapeutic formulation comprising an enzyme as disclosed herein, or the nucleic acid as disclosed herein, in a pharmaceutically acceptable carrier.
- 15 21. A method of treating a subject having or at risk of developing cystinuria comprising administering to the subject a therapeutically effective amount of a formulation of aspect 20.
22. The method as disclosed herein, wherein the subject is maintained on a L-cystine and/or L-cysteine restricted diet.
- 20 23. The method as disclosed herein, wherein the subject is maintained on a methionine-restricted diet.
24. The method as disclosed herein, wherein the subject is maintained on a normal diet.
25. The method as disclosed herein, wherein the subject is a human patient.
- 25 26. The method as disclosed herein, wherein the formulation is administered intravenously, intraarterially, intraperitoneally, intramuscularly, intravascularly, subcutaneously, by injection, by infusion, by continuous infusion, or via a catheter.

27. The method as disclosed herein, wherein the subject has previously been treated for cystinuria and the enzyme is administered to prevent the recurrence of cystinuria.

28. The method as disclosed herein, further comprising administering at least a second cystinuria therapy to the subject.

5 29. The method as disclosed herein, wherein the second cystinuria therapy is a surgical therapy or a shock wave therapy.

30. The method as disclosed herein, wherein the second cystinuria therapy comprises an agent or drug selected from the group consisting of: bicarbonate, tris-hydroxymethylene-aminomethane (tromethamine-E), acetylcysteine, D-penicillamine, α -mercaptopropionylglycine,
10 and captopril.

31. The method as disclosed herein, wherein the method reduces the level of cysteine and/or cystine in the subject's plasma and/or serum.

32. The method as disclosed herein, wherein the method reduces the level of cysteine and/or cystine in the subject's urine.

15 33. The method as disclosed herein, wherein the method prevents the formation of cystine stones in the subject's urinary tract.

34. The method as disclosed herein, wherein the method prevents the formation of cystine stones in the subject's kidneys.

20 35. The method as disclosed herein, wherein the method reduces the number of cystine stones in the subject's kidneys.

36. Use of an isolated or modified CGL enzyme as disclosed herein or a composition comprising said modified CGL enzyme for the manufacture of a medicament for therapeutic application to a cystinuria patient.

25 **[0030]** As used herein the terms "encode" or "encoding," with reference to a nucleic acid, are used to make the disclosure readily understandable by the skilled artisan; however, these terms may be used interchangeably with "comprise" or "comprising," respectively.

[0031] As used herein, “essentially free,” in terms of a specified component, is used herein to mean that none of the specified component has been purposefully formulated into a composition and/or is present only as a contaminant or in trace amounts. The total amount of the specified component resulting from any unintended contamination of a composition is therefore well below 0.05%, preferably below 0.01%. Most preferred is a composition in which no amount of the specified component can be detected with standard analytical methods.

[0032] As used herein the specification, “a” or “an” may mean one or more. As used herein in the claim(s), when used in conjunction with the word “comprising”, the words “a” or “an” may mean one or more than one.

[0033] The use of the term “or” in the claims is used to mean “and/or” unless explicitly indicated to refer to alternatives only or the alternatives are mutually exclusive, although the disclosure supports a definition that refers to only alternatives and “and/or.” As used herein “another” may mean at least a second or more.

[0034] As used here, the term “about” is understood by persons of ordinary skill in the art and will vary to some extent on the context in which it is used. Generally, about encompasses a range of values that are plus/minus 10% of a referenced value.

BRIEF DESCRIPTION OF THE DRAWINGS

[0035] The following drawings form part of the present specification and are included to further exemplify the methods, compounds, and composition described.

[0036] **FIG. 1** – Sequence alignment of SEQ ID NOs: 1-5. Asterisks indicate engineered positions in various of the modified CGL enzymes.

DETAILED DESCRIPTION

[0037] Cysteine is considered a non-essential amino acid as it can be synthesized from homocysteine derived from the essential amino acid L-methionine *via* the transsulfuration pathway, which comprises the enzymes cystathionine- β -synthase (CBS) and cystathionine- γ -lyase (CGL). Thus, the depletion of L-cyst(e)ine is expected to be relatively non-toxic to normal tissues with an intact transsulfuration pathway. Provided are engineered,

therapeutic enzymes that degrade L-cyst(e)ine at a higher k_{cat}/K_M , potentially allowing for use of lower dosages. Also provided are methods of using said enzymes to treat cystinuria.

I. Definitions

[0038] As used herein the terms “enzyme” and “protein” and “polypeptide” refer to
5 compounds comprising amino acids joined *via* peptide bonds and are used interchangeably.

[0039] As used herein, the term “fusion protein” refers to a chimeric protein containing proteins or protein fragments operably linked in a non-native way.

[0040] As used herein, the term “half-life” ($\frac{1}{2}$ -life) refers to the time that would be required for the concentration of a polypeptide to fall by half *in vitro* (i.e., as measured in the
10 cell culture media) or *in vivo* (i.e., as measured in serum), for example, after injection in a mammal. Methods to measure “half-life” include the use of antibodies specific for CGL or PEG used in an ELISA format such that the physical amount of protein is measured as a function of time. Other methods germane to measuring the half-life include determining the catalytic activity of the enzyme drug as a function of time by any assay that detects the
15 production of any substrates resulting from conversion of L-cyst(e)ine, such as the detection of the reaction product pyruvate following derivatization with the agent 3-methyl-2-benzothiazolinone hydrazone (MBTH).

[0041] The terms “in operable combination,” “in operable order,” and “operably linked” refer to a linkage wherein the components so described are in a relationship
20 permitting them to function in their intended manner, for example, a linkage of nucleic acid sequences in such a manner that a nucleic acid molecule capable of directing the transcription of a given gene and/or the synthesis of desired protein molecule, or a linkage of amino acid sequences in such a manner so that a fusion protein is produced.

[0042] The term “linker” is meant to refer to a compound or moiety that acts as a
25 molecular bridge to operably link two different molecules, wherein one portion of the linker is operably linked to a first molecule, and wherein another portion of the linker is operably linked to a second molecule.

[0043] The term “PEGylated” refers to conjugation with polyethylene glycol (PEG), which has been widely used as a drug carrier, given its high degree of biocompatibility and

ease of modification. PEG can be coupled (*e.g.*, covalently linked) to active agents through the hydroxy groups at the end of the PEG chain *via* chemical methods; however, PEG itself is limited to at most two active agents per molecule. In a different approach, copolymers of PEG and amino acids have been explored as novel biomaterial that would retain the biocompatibility of PEG, but that would have the added advantage of numerous attachment points per molecule (thus providing greater drug loading), and that can be synthetically designed to suit a variety of applications.

[0044] The term “gene” refers to a DNA sequence that comprises control and coding sequences necessary for the production of a polypeptide or precursor thereof. The polypeptide can be encoded by a full-length coding sequence or by any portion of the coding sequence so as the desired enzymatic activity is retained.

[0045] The term “native” refers to the typical or wild-type form of a gene, a gene product, or a characteristic of that gene or gene product when isolated from a naturally occurring source. In contrast, the term “modified,” “variant,” “muted,” or “mutant” refers to a gene or gene product that displays modification in sequence and functional properties (*i.e.*, altered characteristics) when compared to the native gene or gene product, wherein the modified gene or gene product is genetically engineered and not naturally present or occurring.

[0046] The term “vector” is used to refer to a carrier nucleic acid molecule into which a nucleic acid sequence can be inserted for introduction into a cell where it can be replicated. A nucleic acid sequence can be “exogenous,” which means that it is foreign to the cell into which the vector is being introduced or that the sequence is homologous to a sequence in the cell but in a position within the host cell nucleic acid in which the sequence is ordinarily not found. Vectors include plasmids, cosmids, viruses (bacteriophage, animal viruses, and plant viruses), and artificial chromosomes (*e.g.*, YACs). One of skill in the art would be well equipped to construct a vector through standard recombinant techniques (*see*, for example, Maniatis *et al.*, 1988 and Ausubel *et al.*, 1994, both incorporated herein by reference).

[0047] The term “expression vector” refers to any type of genetic construct comprising a nucleic acid coding for an RNA capable of being transcribed. In some cases, RNA molecules are then translated into a protein, polypeptide, or peptide. In other cases, these sequences are not translated, for example, in the production of antisense molecules or

ribozymes. Expression vectors can contain a variety of “control sequences,” which refer to nucleic acid sequences necessary for the transcription and possibly translation of an operably linked coding sequence in a particular host cell. In addition to control sequences that govern transcription and translation, vectors and expression vectors may contain nucleic acid sequences that serve other functions as well.

[0048] The term “therapeutically effective amount” as used herein refers to an amount of a therapeutic composition (i.e., a modified CGL enzyme or a nucleic acid encoding such an enzyme) that is employed in methods to achieve a therapeutic effect, *i.e.*, to deplete L-cyst(e)ine in a patient’s circulation to a level of about 0-15 $\mu\text{mol/L}$. The term “therapeutic benefit” or “therapeutically effective” as used throughout this application refers to anything that promotes or enhances the well-being of the subject with respect to the medical treatment of this condition. This includes, but is not limited to, a reduction in the frequency or severity of the signs or symptoms of a disease. For example, treatment of cystinuria may involve a reduction in the concentration of cystine in the urine, reduction in the size of a cystine stone, elimination of a cystine stone, or prevention of the formation of a cystine stone. The dosage ranges for the administration of therapeutic compositions are those large enough to produce the desired effect in which the symptoms of cystinuria are reduced. For example, a therapeutically effective amount of a therapeutic composition may be an amount such that when administered in a physiologically tolerable composition is sufficient to achieve an intravascular (plasma) concentration of from about 0.001 to about 100 units (U) per mL, preferably above about 0.1 U, and more preferably above 1 U modified CGL enzyme per mL. Typical dosages can be administered based on body weight, and are in the range of about 1-100 U/kilogram (kg)/day, preferably about 2-25 U/kg/day, and more preferably about 2-8 U/kg/day. An exemplary amount can be 5 U/kg/day or 35 U/kg/week. Normal human serum L-cyst(e)ine levels are around 200 μM . In the cystinuria patient, serum L-cyst(e)ine levels are reduced to around one half due to lack of renal cystine re-uptake, and dosages would be administered to obtain serum levels of L-cyst(e)ine of about 0-10 μM . The dosage should not be so large as to cause adverse side effects, such as hyperviscosity syndromes, pulmonary edema, congestive heart failure, and the like. Generally, the dosage will vary with age of, condition of, sex of, and extent of the disease in the patient and can be determined by one of skill in the art. The dosage can be adjusted by the individual physician in the event of any complication. The dosage should result in the L-cyst(e)ine content in a subject’s serum being reduced at least by 50%, at least by 60%, and at least by 70% in about 12 to 24 hours.

The dosage could result in the L-cyst(e)ine content in a subject's serum being reduced at least 50% to 70% in 6 hours.

[0049] The term " K_M " as used herein refers to the Michaelis-Menten constant for an enzyme and is defined as the concentration of the specific substrate at which a given enzyme yields one-half its maximum velocity in an enzyme catalyzed reaction. The term " k_{cat} " as used herein refers to the turnover number or the number of substrate molecules each enzyme site converts to product per unit time, and in which the enzyme is working at maximum efficiency. The term " k_{cat}/K_M " as used herein is the specificity constant, which is a measure of how efficiently an enzyme converts a substrate into product.

[0050] The term "cystathionine- γ -lyase" (CGL or cystathionase) refers to any enzyme that catalyzes the γ -elimination of cystathionine to cysteine. As used herein, the terms also contemplate primate forms of cystathionine- γ -lyase (or cystathionine-gamma-lyase), including the human form of cystathionine- γ -lyase.

[0051] "Treatment" and "treating" refer to administration or application of a therapeutic agent to a subject or performance of a procedure or modality on a subject to obtain a therapeutic benefit of a disease or health-related condition. For example, treatment includes administration of a therapeutically effective amount of a modified CGL enzyme in order to reduce serum L-cyst(e)ine levels.

[0052] "Subject" and "patient" refer to either a human or a non-human, such as primates, mammals, and vertebrates.

II. Cystathionine- γ -lyase

[0053] A lyase is an enzyme that catalyzes the breaking of various chemical bonds, often forming a new double bond or a new ring structure. For example, an enzyme that catalyzed this reaction would be a lyase: $ATP \rightarrow cAMP + PP_i$. Certain lyases only require one substrate; for example, cystathionine- γ -lyase converts L-cystathionine to L-cysteine, alpha-ketobutyrate, and ammonia. Other lyases, known as synthases, require two substrates; for example, cystathionine- β -synthase condenses serine and homocysteine to form cystathionine.

[0054] A number of pyrioxal-5'-phosphate (PLP)-dependent enzymes are involved in the metabolism of cysteine, homocysteine, and methionine, and these enzymes form an

evolutionary related family, designated as Cysteine/Methionine (Cys/Met) metabolism PLP-dependent enzymes. These enzymes are proteins of about 400 amino acids and the PLP group forms an internal aldimine with a lysine residue located in the central location of the polypeptide. Members of this family include cystathionine- γ -lyase (CGL), cystathionine- γ -synthase (CGS), cystathionine- β -lyase (CBL), methionine- γ -lyase (MGL), and O-acetylhomoserine (OAH)/O-acetyl-serine (OAS) sulfhydrylase (OSHS). Common to all of the PLP-dependent enzymes is the formation of a Michaelis complex followed by transaldimination of the substrate leading to formation of an external aldimine. The further course of the reaction is determined by the substrate specificity of the particular enzyme.

[0055] For example, the inventors introduced specific mutations into a PLP-dependent lyase family member, cystathionine- γ -lyase, to change its substrate specificity. In this manner, variants were produced with the enhanced ability to degrade both L-cystine and L-cysteine. A modification of other PLP-dependent enzymes for producing novel L-cyst(e)ine degrading activity may also be contemplated.

[0056] CGL is a tetramer that catalyzes the last step in the mammalian transsulfuration pathway (Rao *et al.*, 1990). CGL catalyzes the conversion of L-cystathionine to L-cysteine, alpha-ketobutyrate, and ammonia. Pyridoxal phosphate is a prosthetic group of this enzyme. Protein engineering was used to convert CGL, which has only weak activity for degrading L-cysteine and L-cystine, into an enzyme that can degrade L-cysteine and L-cystine at a high rate (U.S. Pat. No. 9,481,877, which is incorporated herein by reference in its entirety).

III. Cyst(e)inase Engineering

[0057] Due to the undesired effects of immunogenicity seen clinically with the use of non-human protein therapeutics, it is desirable to engineer therapeutically relevant L-cystine and L-cysteine degrading activity into a human enzyme (*i.e.*, engineer a L-cyst(e)inase enzyme that displays high k_{cat} and low K_M values for both L-cystine and L-cysteine and also displaying a favorable specificity towards these two substrates). Humans have an enzyme called cystathionine- γ -lyase (hCGL) whose function is to catalyze the last step in the mammalian transsulfuration pathway (Rao *et al.*, 1990), namely the conversion of L-cystathionine to L-cysteine, α -ketobutyrate, and ammonia. Human CGL can also weakly degrade L-cysteine and its disulfide form, L-cystine, making it an ideal candidate for

engineering. Using structurally- and phylogenetically-guided mutagenesis, along with random mutagenesis approaches, hCGL variants were engineered to efficiently hydrolyze both L-cysteine and L-cystine.

5 [0058] Described are modified CGL enzymes that exhibit at least one functional activity that is comparable to the unmodified CGL enzyme. Modified CGL enzymes include, for example, a protein that possesses an additional advantage, such as the cyst(e)inase enzyme activity, compared to the unmodified CGL enzyme. The unmodified protein or polypeptide may be a native cystathionine- γ -lyase, such as a human cystathionine- γ -lyase.

10 [0059] Determination of activity may be achieved using assays familiar to those of skill in the art, particularly with respect to the protein's activity, and may include for comparison purposes, for example, the use of native and/or recombinant versions of either the modified or unmodified enzymes. For example, wild-type human CGL slowly degrades L-cysteine to pyruvate, ammonia and H₂S, and converts L-cystine to pyruvate, ammonia, and thiocysteine ($k_{cat}/K_M \sim 0.2 \text{ s}^{-1} \text{ mM}^{-1}$ and $\sim 0.8 \text{ s}^{-1} \text{ mM}^{-1}$, respectively). Thiocysteine is further
15 nonenzymatically degraded to L-cysteine and H₂S. Thus, the L-cyst(e)ine degrading activity may be determined by any assay to detect the production of any substrates resulting from the degradation of L-cystine and/or L-cysteine, such as the detection of the reaction product pyruvate following derivatization with the agent 3-methyl-2-benzothiazolinone hydrazone (MBTH) (Takakura *et al.*, 2004).

20 [0060] A modified CGL enzyme, may be identified based on its increase in L-cyst(e)ine degrading activity. For example, substrate recognition sites of the unmodified polypeptide may be identified. This identification may be based on structural analysis or homology analysis. A population of mutants involving modifications of such substrate recognitions sites may be generated. Mutants with increased L-cyst(e)ine degrading activity
25 may be selected from the mutant population. Selection of desired mutants may include methods for the detection of byproducts or products from L-cyst(e)ine degradation.

[0061] Modified CGL enzymes may possess deletions and/or substitutions of amino acids; thus, an enzyme with a deletion, an enzyme with a substitution, and an enzyme with a deletion and a substitution are modified CGL enzymes. These modified CGL enzymes may
30 further include insertions or added amino acids, such as with fusion proteins or proteins with linkers, for example. A "modified deleted CGL enzyme" lacks one or more residues of the

native enzyme, but may possess the specificity and/or activity of the native enzyme. A modified deleted CGL enzyme may also have reduced immunogenicity or antigenicity. An example of a modified deleted CGL enzyme is one that has an amino acid residue deleted from at least one antigenic region, that is, a region of the enzyme determined to be antigenic in a particular organism, such as the type of organism that may be administered the modified CGL enzyme.

[0062] Substitution or replacement CGL enzyme variants may contain the exchange of one amino acid for another at one or more sites within the protein and may be designed to modulate one or more properties of the polypeptide, particularly its effector functions and/or bioavailability. Substitutions may or may not be conservative, that is, one amino acid is replaced with one of similar size and charge. Conservative substitutions are well known in the art and include, for example, the changes of: alanine to serine; arginine to lysine; asparagine to glutamine or histidine; aspartate to glutamate; cysteine to serine; glutamine to asparagine; glutamate to aspartate; glycine to proline; histidine to asparagine or glutamine; isoleucine to leucine or valine; leucine to valine or isoleucine; lysine to arginine; methionine to leucine or isoleucine; phenylalanine to tyrosine, leucine, or methionine; serine to threonine; threonine to serine; tryptophan to tyrosine; tyrosine to tryptophan or phenylalanine; and valine to isoleucine or leucine.

[0063] The term “biologically functional equivalent” is well understood in the art and is further defined in detail herein. Accordingly, CGL enzyme sequences that have about 90% or more sequence identity to SEQ ID NO: 1, or even between about 91% and about 99% of amino acids (including 92%, 93%, 94%, 95%, 96%, 97%, 98%, and 99%) that are identical to or conservative substitution of the amino acids of an modified CGL enzyme disclosed herein are included, provided the biological activity of the enzyme is maintained such that a measureable biological activity parameter (e.g., conversion of L-cystine and/or L-cysteine to pyruvate) is within about 20%, about 15%, about 10%, or about 5% of a modified CGL enzyme disclosed herein. A modified CGL enzyme may be biologically functionally equivalent to its unmodified counterpart.

[0064] Amino acid and nucleic acid sequences may include additional residues, such as additional N- or C-terminal amino acids or 5' or 3' sequences, and yet still be essentially as set forth in one of the sequences disclosed herein, so long as the sequence meets the criteria set forth above, including the maintenance of biological protein activity where protein

expression is concerned. The addition of terminal sequences particularly applies to nucleic acid sequences that may, for example, include various non-coding sequences flanking either of the 5' or 3' portions of the coding region or may include various internal sequences, *i.e.*, introns, which are known to occur within genes.

5 IV. Enzymatic L-cyst(e)ine Degradation for Therapy

[0065] Cystinuria is a hereditary disorder caused by mutations in the *SLC3A1* and *SLC7A9* genes encoding the kidney proximal tubule's cystine and dibasic amino acid transporter that leads to abnormal excretion of cystine (the disulfide form of the amino acid cysteine) and the formation of cystine crystals/stones in the urinary tract due to low solubility
10 of cystine.

[0066] Several mouse models of cystinuria are available, including a *Slc7a9* knockout mouse, a *Slc3a1* knockout mouse, a D140G *Slc3a1* mutant mouse, and a E383K *Slc3a1* mutant mouse (Feliubadalo *et al.*, 2003; Ercolani *et al.*, 2010; Peters *et al.*, 2003; Livrozet *et al.*, 2014, each of which is incorporated herein by reference in its entirety). Sequence analysis
15 of *Slc3a1* genomic DNA from 129S2/SvPasCrl revealed a homozygous mutation in exon 7 in 129S2/SvPasCrl mice. The A1232G point mutation is a missense mutation (c.1232G>A) in a highly conserved sequence. As a consequence of the A1232G missense mutation, the glutamine in position 383 is substituted for a lysine (E383K). This substitution stands in the extracellular part of rBAT and is responsible for the loss of rBAT expression and cystinuria
20 in 129S2/SvPasCrl mice (Livrozet *et al.*, 2014). The glutamine in position 383 is highly conserved among various species.

[0067] Patients with cystinuria have a low quality of life, a life-long risk of cystine stone formation, impaired renal function and often require repeated surgical interventions. There are few therapeutics available to patients suffering from the hereditary disorder
25 cystinuria wherein a defective kidney transporter is unable to re-uptake cystine during renal filtration. Cystine, the disulfide form of the amino acid L-cysteine, is highly insoluble and in cystinuria patients reaches high concentrations in the urinary tract resulting in the formation of cystine crystals and stones. Existing therapies that reduce circulating cystine levels partially prevent urinary tract stone formation but have significant adverse effects that limit
30 their use.

[0068] There are no existing curative therapies for cystinuria and treatments are directed at increasing cystine solubility and lowering urinary cystine concentrations. Hyperdiuresis is a common treatment; however it requires daily consumption of >4 liters of water and urine volumes >3 liters which is difficult to achieve and maintain. Other drug treatments, such as small thiol molecules, function by reacting with cystine to form mixed disulfides that are more soluble than cystine but have significant toxicities, such as leukopenia, rash, fever, proteinuria and nephritic syndrome, which limit their use.

[0069] The present invention provides methods of using engineered, therapeutic enzymes that degrade L-cyst(e)ine to treat diseases, such as cystinuria. This method removes cystine from circulation, which has been shown clinically to reduce the incidence of kidney and urinary cystine stone formation in cystinuria patients. The method described here can reduce circulating cystine below detection levels without the side-effects associated with current cystinuria drugs.

[0070] Cysteine is considered a non-essential amino acid as it can be synthesized from homocysteine derived from the essential amino acid L-methionine *via* the transsulfuration pathway, which comprises the enzymes cystathionine- β -synthase (CBS) and cystathionine- γ -lyase (CGL). Thus, the depletion of cysteine is expected to be relatively non-toxic to normal tissues with an intact transsulfuration pathway.

[0071] The polypeptides may be used for the treatment of diseases, such as cystinuria, with novel enzymes that deplete L-cystine and/or L-cysteine. Disclosed are treatment methods using modified CGL with L-cyst(e)ine degrading activity. Enzymes with L-cyst(e)ine degrading activity for increased therapeutic efficacy are provided.

[0072] Provided are modified CGL enzymes with L-cyst(e)ine degrading activity for treating cystinuria. A modified polypeptide may have human polypeptide sequences and thus may prevent adverse immunogenic reactions when administered to human patients, allow repeated dosing, and increase the therapeutic efficacy.

[0073] As an example, PEG-hCGL-TV can drastically reduce serum cystine levels (> 95%) for over 96 h and cysteine levels (80%) for over 48 h in a murine model. To determine this, male FVB/N mice of 6-7 weeks of age (JAX 001800) were injected intraperitoneally (i.p.) with 50 mg/kg of PEG-hCGL-TV and sacrificed at days 0, 1, 2, 4, and 6 (n = 5 per group) for blood and serum collection. Serum samples were mixed with an internal standard

mixture of 10 picomole deuterated cystine and cysteine and ultrafiltered using NANOSEP® OMEGA™ centrifugal devices, 3 kDa cutoff (Pall Life Biosciences) (Tiziani *et al.*, 2008; Tiziani *et al.*, 2013). The filtered polar fractions were chromatographed using a reverse-phase BEH C18, 1.7 µm, 2.1 × 150 mm column (THERMO SCIENTIFIC™ ACCELA® 1250 UPLC, Waters Corporation, USA) and introduced into an EXACTIVE™ Plus ORBITRAP™ mass spectrometer coupled with electrospray ionization (Thermo Fisher Scientific, San Jose, CA) according to manufacturer instructions. Data were acquired in centroid MS mode from 50 to 700 m/z mass range with the XCALIBUR™ software provided with instrument.

[0074] In addition, PEG-hCGL-TV demonstrated an absorption $T_{1/2}$ of approximately 23 h, and an elimination $T_{1/2}$ of 40 ± 7 h. To determine this, a dot blot densitometry technique was used where appropriate serum samples were probed with an anti-hCGL antibody (rabbit anti-CTH Sigma # C8248) followed by addition of anti-rabbit IgG-fluorescein isothiocyanate (FITC) (Santa Cruz Biotechnology # sc-2012) and visualization by excitation at 488 nm on a TYPHOON™ scanner (GE Healthcare). Using ImageJ software (Schneider *et al.*, 2012), densitometry of samples on the scanned dot blots were compared to titrations of known amounts of PEG-hCGL-TV within the same blot to construct a standard curve and calculate relative serum PEG-hCGL-TV levels. The data were fit to an extravascular model of administration (Foye *et al.*, 2007).

[0075] Depletion can be conducted *in vivo* in the circulation of a mammal, *in vitro* in cases where L-cystine and/or L-cysteine depletion in tissue culture or other biological mediums is desired, and in *ex vivo* procedures where biological fluids, cells, or tissues are manipulated outside the body and subsequently returned to the body of the patient mammal. Depletion of L-cystine and/or L-cysteine from circulation, culture media, biological fluids, or cells is conducted to reduce the amount of L-cystine and/or L-cysteine accessible to the material being treated, and therefore comprises contacting the material to be depleted with a L-cystine- and/or L-cysteine-degrading amount of the engineered enzyme under L-cystine- and/or L-cysteine-degrading conditions as to degrade the ambient L-cystine and/or L-cysteine in the material being contacted.

[0076] L-cystine- and/or L-cysteine-degrading efficiency can vary widely depending upon the application, and typically depends upon the amount of L-cystine and/or L-cysteine present in the material, the desired rate of depletion, and the tolerance of the material for exposure to cyst(e)inase. L-cystine and/or L-cysteine levels in a material, and therefore rates

of L-cystine and/or L-cysteine depletion from the material, can readily be monitored by a variety of chemical and biochemical methods well known in the art. Exemplary L-cystine- and/or L-cysteine-degrading amounts are described further herein, and can range from 0.001 to 100 units (U) of engineered cyst(e)inase, preferably about 0.01 to 10 U, and more preferably about 0.1 to 5 U engineered cyst(e)inase per milliliter (mL) of material to be treated.

[0077] L-cystine- and/or L-cysteine-degrading conditions are buffer and temperature conditions compatible with the biological activity of a CGL enzyme, and include moderate temperature, salt, and pH conditions compatible with the enzyme, for example, physiological conditions. Exemplary conditions include about 4-40°C., ionic strength equivalent to about 0.05 to 0.2 M NaCl, and a pH of about 5 to 9, while physiological conditions are included.

[0078] The contacting *in vivo* is accomplished by administering, by intravenous or intraperitoneal injection, a therapeutically effective amount of a physiologically tolerable composition comprising modified CGL enzyme to a patient, thereby depleting the circulating L-cystine and/or L-cysteine present in the patient.

[0079] The modified CGL enzyme can be administered parenterally by injection or by gradual infusion over time. The modified CGL enzyme can be administered intravenously, intraperitoneally, intramuscularly, subcutaneously, or can be administered by a pump connected to a catheter that may contain a potential biosensor for L-cyst(e)ine. For cystinuria, it may be desirable to administer the CGL enzymes subcutaneously.

[0080] The therapeutic compositions containing modified CGL enzyme are conventionally administered intravenously, as by injection of a unit dose, for example. The term “unit dose” when used in reference to a therapeutic composition refers to physically discrete units suitable as unitary dosage for the subject, each unit containing a predetermined quantity of active material calculated to produce the desired therapeutic effect in association with the required diluent, *i.e.*, carrier, or vehicle.

[0081] The compositions are administered in a manner compatible with the dosage formulation, and in a therapeutically effective amount. The quantity to be administered depends on the subject to be treated, capacity of the subject’s system to utilize the active ingredient, and degree of therapeutic effect desired. Precise amounts of active ingredient required to be administered depend on the judgment of the practitioner and are peculiar to

each individual. However, suitable dosage ranges for systemic application are disclosed herein and depend on the route of administration. Suitable regimes for initial administration and booster shots are also contemplated and are typified by an initial administration followed by repeated doses at one or more hour intervals by a subsequent injection or other administration. Exemplary multiple administrations are described herein and are particularly preferred to maintain continuously high serum and tissue levels of modified CGL enzyme and conversely low serum and tissue levels of L-cyst(e)ine. Alternatively, continuous intravenous infusion sufficient to maintain concentrations in the blood in the ranges specified for *in vivo* therapies are contemplated.

V. Conjugates

[0082] The compositions and methods provided involve further modification of the modified CGL enzyme for improvement, such as by forming conjugates with heterologous peptide segments or polymers, such as polyethylene glycol. The modified CGL enzyme may be linked to PEG to increase the hydrodynamic radius of the enzyme and hence increase the serum persistence or half-life. The disclosed polypeptide may be conjugated to any targeting agent, such as a ligand having the ability to specifically and stably bind to an external receptor or binding site on a cell (U.S. Patent Publ. 2009/0304666). The PEG can be from about 3,000 to 20,000 Daltons in size, with an exemplary size being 5,000 Daltons.

A. Fusion Proteins

[0083] Fusion proteins are provided in which the modified CGL enzyme may be linked at the N- or C-terminus to a heterologous domain. For example, fusions may also employ leader sequences from other species to permit the recombinant expression of a protein in a heterologous host. Another useful fusion includes the addition of a protein affinity tag, such as a serum albumin affinity tag or six histidine residues, or an immunologically active domain, such as an antibody epitope, preferably cleavable, to facilitate purification of the fusion protein. Non-limiting affinity tags include polyhistidine, chitin binding protein (CBP), maltose binding protein (MBP), and glutathione-S-transferase (GST).

[0084] The modified CGL enzyme may be linked to a peptide that increases the *in vivo* half-life, such as an XTEN polypeptide (Schellenberger *et al.*, 2009), IgG Fc domain, albumin, or an albumin binding peptide.

[0085] Methods of generating fusion proteins are well known to those of skill in the art. Such proteins can be produced, for example, by *de novo* synthesis of the complete fusion protein, or by attachment of the DNA sequence encoding the heterologous domain, followed by expression of the intact fusion protein.

5 [0086] Production of fusion proteins that recover the functional activities of the parent proteins may be facilitated by connecting genes with a bridging DNA segment encoding a peptide linker that is spliced between the polypeptides connected in tandem. The linker would be of sufficient length to allow proper folding of the resulting fusion protein.

B. Linkers

10 [0087] The modified CGL enzyme may be chemically conjugated using bifunctional cross-linking reagents or fused at the protein level with peptide linkers. Bifunctional cross-linking reagents have been extensively used for a variety of purposes, including preparation of affinity matrices, modification and stabilization of diverse structures, identification of ligand and receptor binding sites, and structural studies. Suitable peptide linkers may also be
15 used to link the modified CGL enzyme, such as Gly-Ser linkers.

 [0088] Homobifunctional reagents that carry two identical functional groups may induce cross-linking between identical and different macromolecules or subunits of a macromolecule, and link polypeptide ligands to their specific binding sites. Heterobifunctional reagents contain two different functional groups. By taking advantage of
20 the differential reactivities of the two different functional groups, cross-linking can be controlled both selectively and sequentially. The bifunctional cross-linking reagents can be divided according to the specificity of their functional groups, *e.g.*, amino-, sulfhydryl-, guanidine-, indole-, carboxyl-specific groups. Of these, reagents directed to free amino groups have become popular because of their commercial availability, ease of synthesis, and
25 the mild reaction conditions under which they can be applied.

 [0089] Some heterobifunctional cross-linking reagents contain a primary amine-reactive group and a thiol-reactive group. In another example, heterobifunctional cross-linking reagents and methods of using the cross-linking reagents are described (U.S. Pat. No. 5,889,155, specifically incorporated herein by reference in its entirety). The cross-linking
30 reagents combine a nucleophilic hydrazide residue with an electrophilic maleimide residue,

allowing coupling, in one example, of aldehydes to free thiols. The cross-linking reagent can be modified to cross-link various functional groups.

[0090] Additionally, any other linking/coupling agents and/or mechanisms known to those of skill in the art may be used to combine modified CGL enzymes, such as, for example, antibody-antigen interaction, avidin biotin linkages, amide linkages, ester linkages, thioester linkages, ether linkages, thioether linkages, phosphoester linkages, phosphoramidate linkages, anhydride linkages, disulfide linkages, ionic and hydrophobic interactions, bispecific antibodies and antibody fragments, or combinations thereof.

[0091] It is preferred that a cross-linker having reasonable stability in blood will be employed. Numerous types of disulfide-bond containing linkers are known that can be successfully employed to conjugate targeting and therapeutic/preventative agents. Linkers that contain a disulfide bond that is sterically hindered may prove to give greater stability *in vivo*. These linkers are thus one group of linking agents.

[0092] In addition to hindered cross-linkers, non-hindered linkers also can be employed. Other useful cross-linkers, not considered to contain or generate a protected disulfide, include SATA, SPDP, and 2-iminothiolane (Wawrzynczak and Thorpe, 1987). The use of such cross-linkers is well understood in the art. Flexible linkers may also be used.

[0093] Once chemically conjugated, the peptide generally will be purified to separate the conjugate from unconjugated agents and from other contaminants. A large number of purification techniques are available for use in providing conjugates of a sufficient degree of purity to render them clinically useful.

[0094] Purification methods based upon size separation, such as gel filtration, gel permeation, or high performance liquid chromatography, will generally be of most use. Other chromatographic techniques, such as Blue-Sepharose separation, may also be used. Conventional methods to purify the fusion proteins from inclusion bodies may be useful, such as using weak detergents, such as sodium N-lauroyl-sarcosine (SLS).

C. PEGylation

[0095] Methods and compositions related to PEGylation of modified CGL enzyme are disclosed. For example, the modified CGL enzyme may be PEGylated in accordance with the methods disclosed herein.

5 [0096] PEGylation is the process of covalent attachment of poly(ethylene glycol) polymer chains to another molecule, normally a drug or therapeutic protein. PEGylation is routinely achieved by incubation of a reactive derivative of PEG with the target macromolecule. The covalent attachment of PEG to a drug or therapeutic protein can “mask” the agent from the host's immune system (reduced immunogenicity and antigenicity) or
10 increase the hydrodynamic size (size in solution) of the agent, which prolongs its circulatory time by reducing renal clearance. PEGylation can also provide water solubility to hydrophobic drugs and proteins.

[0097] The first step of the PEGylation is the suitable functionalization of the PEG polymer at one or both terminals. PEGs that are activated at each terminus with the same
15 reactive moiety are known as “homobifunctional,” whereas if the functional groups present are different, then the PEG derivative is referred as “heterobifunctional” or “heterofunctional.” The chemically active or activated derivatives of the PEG polymer are prepared to attach the PEG to the desired molecule.

[0098] The choice of the suitable functional group for the PEG derivative is based on
20 the type of available reactive group on the molecule that will be coupled to the PEG. For proteins, typical reactive amino acids include lysine, cysteine, histidine, arginine, aspartic acid, glutamic acid, serine, threonine, and tyrosine. The N-terminal amino group and the C-terminal carboxylic acid can also be used.

[0099] The techniques used to form first generation PEG derivatives are generally
25 reacting the PEG polymer with a group that is reactive with hydroxyl groups, typically anhydrides, acid chlorides, chloroformates, and carbonates.

[00100] As applications of PEGylation have become more and more advanced and sophisticated, there has been an increase in need for heterobifunctional PEGs for conjugation. These heterobifunctional PEGs are very useful in linking two entities, where a
30 hydrophilic, flexible, and biocompatible spacer is needed. Preferred end groups for

heterobifunctional PEGs can be maleimide, vinyl sulfones, pyridyl disulfide, amine, carboxylic acids, and NHS esters.

[00101] The most common modification agents, or linkers, are based on methoxy PEG (mPEG) molecules. Their activity depends on adding a protein-modifying group to the alcohol end. In some instances, polyethylene glycol (PEG diol) is used as the precursor molecule. The diol is subsequently modified at both ends in order to make a hetero- or homo-dimeric PEG-linked molecule.

[00102] Proteins are generally PEGylated at nucleophilic sites, such as unprotonated thiols (cysteinyll residues) or amino groups. Examples of cysteinyll-specific modification reagents include PEG maleimide, PEG iodoacetate, PEG thiols, and PEG vinylsulfone. All four are strongly cysteinyll-specific under mild conditions and neutral to slightly alkaline pH but each has some drawbacks. The thioether formed with the maleimides can be somewhat unstable under alkaline conditions so there may be some limitation to formulation options with this linker. The carbamothioate linkage formed with iodo PEGs is more stable, but free iodine can modify tyrosine residues under some conditions. PEG thiols form disulfide bonds with protein thiols, but this linkage can also be unstable under alkaline conditions. PEG-vinylsulfone reactivity is relatively slow compared to maleimide and iodo PEG; however, the thioether linkage formed is quite stable. Its slower reaction rate also can make the PEG-vinylsulfone reaction easier to control.

[00103] Site-specific PEGylation at native cysteinyll residues is seldom carried out, since these residues are usually in the form of disulfide bonds or are required for biological activity. On the other hand, site-directed mutagenesis can be used to incorporate cysteinyll PEGylation sites for thiol-specific linkers. The cysteine mutation must be designed such that it is accessible to the PEGylation reagent and is still biologically active after PEGylation.

[00104] Amine-specific modification agents include PEG NHS ester, PEG tresylate, PEG aldehyde, PEG isothiocyanate, and several others. All react under mild conditions and are very specific for amino groups. The PEG NHS ester is probably one of the more reactive agents; however, its high reactivity can make the PEGylation reaction difficult to control on a large scale. PEG aldehyde forms an imine with the amino group, which is then reduced to a secondary amine with sodium cyanoborohydride. Unlike sodium borohydride,

sodium cyanoborohydride will not reduce disulfide bonds. However, this chemical is highly toxic and must be handled cautiously, particularly at lower pH where it becomes volatile.

[00105] Due to the multiple lysine residues on most proteins, site-specific PEGylation can be a challenge. Fortunately, because these reagents react with unprotonated amino groups, it is possible to direct the PEGylation to lower-pK amino groups by performing the reaction at a lower pH. Generally, the pK of the alpha-amino group is 1-2 pH units lower than the epsilon-amino group of lysine residues. By PEGylating the molecule at pH 7 or below, high selectivity for the N-terminus frequently can be attained. However, this is only feasible if the N-terminal portion of the protein is not required for biological activity. Still, the pharmacokinetic benefits from PEGylation frequently outweigh a significant loss of *in vitro* bioactivity, resulting in a product with much greater *in vivo* bioactivity regardless of PEGylation chemistry.

[00106] There are several parameters to consider when developing a PEGylation procedure. Fortunately, there are usually no more than four or five parameters. The “design of experiments” approach to optimization of PEGylation conditions can be very useful. For thiol-specific PEGylation reactions, parameters to consider include: protein concentration, PEG-to-protein ratio (on a molar basis), temperature, pH, reaction time, and in some instances, the exclusion of oxygen. (Oxygen can contribute to intermolecular disulfide formation by the protein, which will reduce the yield of the PEGylated product.) The same factors should be considered (with the exception of oxygen) for amine-specific modification except that pH may be even more critical, particularly when targeting the N-terminal amino group.

[00107] For both amine- and thiol-specific modifications, the reaction conditions may affect the stability of the protein. This may limit the temperature, protein concentration, and pH. In addition, the reactivity of the PEG linker should be known before starting the PEGylation reaction. For example, if the PEGylation agent is only 70% active, the amount of PEG used should ensure that only active PEG molecules are counted in the protein-to-PEG reaction stoichiometry.

VI. Proteins and Peptides

[00108] Compositions comprising at least one protein or peptide, such as a modified CGL enzyme, are provided. These peptides may be comprised in a fusion protein or conjugated to an agent as described supra.

5 [00109] As used herein, a protein or peptide generally refers, but is not limited to, a protein of greater than about 200 amino acids, up to a full-length sequence translated from a gene; a polypeptide of greater than about 100 amino acids; and/or a peptide of from about 3 to about 100 amino acids. For convenience, the terms “protein,” “polypeptide,” and “peptide” are used interchangeably herein.

10 [00110] Accordingly, the term “protein or peptide” encompasses amino acid sequences comprising at least one of the 20 common amino acids found in naturally occurring proteins, or at least one modified or non-natural amino acid.

[00111] Proteins or peptides may be made by any technique known to those of skill in the art, including the expression of proteins, polypeptides, or peptides through
15 standard molecular biological techniques, the isolation of proteins or peptides from natural sources, or the chemical synthesis of proteins or peptides. The coding regions for known genes may be amplified and/or expressed using the techniques disclosed herein or as would be known to those of ordinary skill in the art. Alternatively, various commercial preparations of proteins, polypeptides, and peptides are known to those of skill in the art.

20 VII. Nucleic Acids and Vectors

[00112] Nucleic acid sequences encoding a modified CGL enzyme or a fusion protein containing a modified CGL enzyme are disclosed. Depending on which expression system is used, nucleic acid sequences can be selected based on conventional methods. For example, if the modified CGL enzyme is derived from human cystathionase and contains
25 multiple codons that are rarely utilized in *E. coli*, then that may interfere with expression. Therefore, the respective genes or variants thereof may be codon optimized for *E. coli* expression using freely available software (*see* Hoover & Lubkowski, 2002) to design coding sequences free of rare codons. Various vectors may be also used to express the protein of interest, such as a modified CGL enzyme. Exemplary vectors include, but are not limited,
30 plasmid vectors, viral vectors, transposon, or liposome-based vectors.

VIII. Host Cells

[00113] Host cells may be any that may be transformed to allow the expression and secretion of modified CGL enzyme and conjugates thereof. The host cells may be bacteria, mammalian cells, yeast, or filamentous fungi. Various bacteria include *Escherichia* and *Bacillus*. Yeasts belonging to the genera *Saccharomyces*, *Kluyveromyces*, *Hansenula*, or *Pichia* would find use as an appropriate host cell. Various species of filamentous fungi may be used as expression hosts, including the following genera: *Aspergillus*, *Trichoderma*, *Neurospora*, *Penicillium*, *Cephalosporium*, *Achlya*, *Podospora*, *Endothia*, *Mucor*, *Cochliobolus*, and *Pyricularia*.

[00114] Examples of usable host organisms include bacteria, *e.g.*, *Escherichia coli* MC1061, derivatives of *Bacillus subtilis* BRB1 (Sibakov *et al.*, 1984), *Staphylococcus aureus* SAI123 (Lordanescu, 1975) or *Streptococcus lividans* (Hopwood *et al.*, 1985); yeasts, *e.g.*, *Saccharomyces cerevisiae* AH 22 (Mellor *et al.*, 1983) or *Schizosaccharomyces pombe*; and filamentous fungi, *e.g.*, *Aspergillus nidulans*, *Aspergillus awamori* (Ward, 1989), or *Trichoderma reesei* (Penttila *et al.*, 1987; Harkki *et al.*, 1989).

[00115] Examples of mammalian host cells include Chinese hamster ovary cells (CHO-K1; American Type Culture Collection (ATCC) No. CCL61), rat pituitary cells (GH1; ATCC No. CCL82), HeLa S3 cells (ATCC No. CCL2.2), rat hepatoma cells (H-4-II-E; ATCC No. CRL-1548), SV40-transformed monkey kidney cells (COS-1; ATCC No. CRL-1650), and murine embryonic cells (NIH-3T3; ATCC No. CRL-1658). The foregoing is meant to be illustrative but not limitative of the many possible host organisms known in the art. In principle, all hosts capable of secretion can be used whether prokaryotic or eukaryotic.

[00116] Mammalian host cells expressing the modified CGL enzymes and/or their fusion proteins are cultured under conditions typically employed to culture the parental cell line. Generally, cells are cultured in a standard medium containing physiological salts and nutrients, such as standard RPMI, MEM, IMEM, or DMEM, typically supplemented with 5%-10% serum, such as fetal bovine serum or as described for the desired cell. Culture conditions are also standard, *e.g.*, cultures are incubated at 37 °C in stationary or roller cultures until desired levels of the proteins are achieved.

IX. Protein Purification

[00117] Protein purification techniques are well known to those of skill in the art. These techniques involve, at one level, the homogenization and crude fractionation of the cells, tissue, or organ to polypeptide and non-polypeptide fractions. The protein or polypeptide of interest may be further purified using chromatographic and electrophoretic techniques to achieve partial or complete purification (or purification to homogeneity) unless otherwise specified. Analytical methods particularly suited to the preparation of a pure peptide are ion-exchange chromatography, gel exclusion chromatography, polyacrylamide gel electrophoresis, affinity chromatography, immunoaffinity chromatography, and isoelectric focusing. A particularly efficient method of purifying peptides is fast-performance liquid chromatography (FPLC) or even high-performance liquid chromatography (HPLC).

[00118] A purified protein or peptide is intended to refer to a composition, isolatable from other components, wherein the protein or peptide is purified to any degree relative to its naturally-obtainable state. An isolated or purified protein or peptide, therefore, also refers to a protein or peptide free from the environment in which it may naturally occur. Generally, “purified” will refer to a protein or peptide composition that has been subjected to fractionation to remove various other components, and which composition substantially retains its expressed biological activity. Where the term “substantially purified” is used, this designation will refer to a composition in which the protein or peptide forms the major component of the composition, such as constituting about 50%, about 60%, about 70%, about 80%, about 90%, about 95%, or more of the proteins in the composition.

[00119] Various techniques suitable for use in protein purification are well known to those of skill in the art. These include, for example, precipitation with ammonium sulfate, PEG, antibodies and the like, or by heat denaturation, followed by centrifugation; chromatography steps, such as ion exchange, gel filtration, reverse phase, hydroxyapatite, and affinity chromatography; isoelectric focusing; gel electrophoresis; and combinations of these and other techniques. As is generally known in the art, it is believed that the order of conducting the various purification steps may be changed, or that certain steps may be omitted, and still result in a suitable method for the preparation of a substantially purified protein or peptide.

[00120] Various methods for quantifying the degree of purification of the protein or peptide are known to those of skill in the art. These include, for example, determining the specific activity of an active fraction, or assessing the amount of polypeptides within a fraction by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS/PAGE) analysis. A preferred method for assessing the purity of a fraction is to calculate the specific activity of the fraction, to compare it to the specific activity of the initial extract, and to thus calculate the degree of purity therein, assessed by a “-fold purification number.” The actual units used to represent the amount of activity will, of course, be dependent upon the particular assay technique chosen to follow the purification, and whether or not the expressed protein or peptide exhibits a detectable activity.

[00121] There is no general requirement that the protein or peptide will always be provided in its most purified state. Indeed, it is contemplated that less substantially purified products may have utility. Partial purification may be accomplished by using fewer purification steps in combination, or by utilizing different forms of the same general purification scheme. For example, it is appreciated that a cation-exchange column chromatography performed utilizing a high-performance liquid chromatography (HPLC) apparatus will generally result in a greater “-fold” purification than the same technique utilizing a low pressure chromatography system. Methods exhibiting a lower degree of relative purification may have advantages in total recovery of protein product, or in maintaining the activity of an expressed protein.

[00122] A protein or peptide may be isolated or purified, for example, a modified CGL enzyme, a fusion protein containing the modified CGL enzyme, or a modified CGL enzyme post PEGylation. For example, a His tag or an affinity epitope may be comprised in such a modified CGL enzyme to facilitate purification. Affinity chromatography is a chromatographic procedure that relies on the specific affinity between a substance to be isolated and a molecule to which it can specifically bind. This is a receptor-ligand type of interaction. The column material is synthesized by covalently coupling one of the binding partners to an insoluble matrix. The column material is then able to specifically adsorb the substance from the solution. Elution occurs by changing the conditions to those in which binding will not occur (*e.g.*, altered pH, ionic strength, temperature, *etc.*). The matrix should be a substance that does not adsorb molecules to any significant extent and that has a broad range of chemical, physical, and thermal stability. The ligand should be coupled in

such a way as to not affect its binding properties. The ligand should also provide relatively tight binding. It should be possible to elute the substance without destroying the sample or the ligand.

[00123] Size exclusion chromatography (SEC) is a chromatographic method in which molecules in solution are separated based on their size, or in more technical terms, their hydrodynamic volume. It is usually applied to large molecules or macromolecular complexes, such as proteins and industrial polymers. Typically, when an aqueous solution is used to transport the sample through the column, the technique is known as gel filtration chromatography, versus the name gel permeation chromatography, which is used when an organic solvent is used as a mobile phase.

[00124] The underlying principle of SEC is that particles of different sizes will elute (filter) through a stationary phase at different rates. This results in the separation of a solution of particles based on size. Provided that all the particles are loaded simultaneously or near simultaneously, particles of the same size should elute together. Each size exclusion column has a range of molecular weights that can be separated. The exclusion limit defines the molecular weight at the upper end of this range and is where molecules are too large to be trapped in the stationary phase. The permeation limit defines the molecular weight at the lower end of the range of separation and is where molecules of a small enough size can penetrate into the pores of the stationary phase completely and all molecules below this molecular mass are so small that they elute as a single band.

[00125] High-performance liquid chromatography (or high-pressure liquid chromatography, HPLC) is a form of column chromatography used frequently in biochemistry and analytical chemistry to separate, identify, and quantify compounds. HPLC utilizes a column that holds chromatographic packing material (stationary phase), a pump that moves the mobile phase(s) through the column, and a detector that shows the retention times of the molecules. Retention time varies depending on the interactions between the stationary phase, the molecules being analyzed, and the solvent(s) used.

X. Therapeutic Compositions

[00126] The human cystathionine- γ -lyase gene contains multiple codons that are rarely utilized in *E. coli* and can interfere with expression. Thus, in order to optimize protein expression in *E. coli*, the respective genes may be assembled with codon optimized

oligonucleotides designed using DNA-Works software (Hoover *et al.*, 2002). Each construct may contain an N-terminal *NcoI* restriction site, an in-frame N-terminal His₆ tag, and a C-terminal *EcoRI* site for simplifying cloning. After cloning into a pET28a vector (Novagen), *E. coli* (BL21) containing an appropriate modified CGL expression vector may be grown at 37 °C using Terrific Broth (TB) media containing 50 µg/mL kanamycin in shaker flasks at 250 rpm until reaching an OD₆₀₀ of ~ 0.5-0.6. At this point the cultures may be switched to a shaker at 25 °C, induced with 0.5 mM IPTG, and allowed to express protein for an additional 12 h. Cell pellets may be then collected by centrifugation and re-suspended in an IMAC buffer (10 mM NaPO₄/10 mM imidazole/300 mM NaCl, pH 8). After lysis by means of a French pressure cell press or by a high pressure homogenizer, lysates may be centrifuged at 20,000 x g for 20 min at 4 °C, and the resulting supernatant applied to a nickel IMAC column (bead size 45-165 µm, Qiagen), washed extensively (90-100 column volumes) with an IMAC buffer containing 0.1% TRITON® 114, washed with 10-20 column volumes of IMAC buffer, and then eluted with an IMAC elution buffer (50 mM NaPO₄/250 mM imidazole/300 mM NaCl, pH 8). The purified protein was subjected to buffer exchange into a 100 mM NaPO₄ buffer at pH 8.3 using a 10,000 MWCO (molecule weight cut off) filtration device (Amicon). Fractions containing enzyme may be then incubated with 10 mM pyridoxal-5'-phosphate (PLP) for an hour at 25 °C. Methoxy PEG succinimidyl carboxymethyl ester 5000 MW (JenKem Technology) was then added to modified CGL enzyme at an 80:1 molar ratio and allowed to react for 1 h at 25 °C under constant stirring. The resulting mixture was extensively buffer exchanged (PBS with 10% glycerol) using a 100,000 MWCO filtration device (Amicon), and sterilized with a 0.2 micron syringe filter (VWR). Enzyme aliquots may be then flash frozen in liquid nitrogen and stored at -80 °C. The modified CGL enzyme variants purified in this manner should be >95% homogeneous as assessed by SDS-PAGE and Coomassie staining. The yield may be calculated based upon the calculated extinction coefficient, $\lambda_{280} = 29,870 \text{ M}^{-1}\text{cm}^{-1}$ in a final buffer concentration of 6 M guanidinium hydrochloride, 20 mM phosphate buffer, pH 6.5 (Gill and von Hippel, 1989). PEGylated modified CGL enzymes may be analyzed for lipopolysaccharide (LPS) content using a Limulus Amebocyte Lysate (LAL) kit.

[00127] No limitation as to the particular nature of the therapeutic preparation is intended. For example, such compositions can be provided in formulations together with physiologically tolerable liquid, gel, or solid carriers, diluents, and excipients. These therapeutic preparations can be administered to mammals for veterinary use, such as with

domestic animals, and clinical use in humans in a manner similar to other therapeutic agents. In general, the dosage required for therapeutic efficacy will vary according to the type of use and mode of administration, as well as the particularized requirements of individual subjects.

[00128] Such compositions are typically prepared as liquid solutions or
5 suspensions, for use as injectables. Suitable diluents and excipients are, for example, water, saline, dextrose, glycerol, or the like, and combinations thereof. In addition, if desired, the compositions may contain minor amounts of auxiliary substances, such as wetting or emulsifying agents, stabilizing agents, or pH buffering agents.

[00129] Where clinical applications are contemplated, it may be necessary to
10 prepare therapeutic compositions comprising proteins, antibodies, and drugs in a form appropriate for the intended application. Generally, therapeutic compositions may comprise an effective amount of one or more modified CGL enzymes or additional agents dissolved or dispersed in a pharmaceutically acceptable carrier. The phrases “therapeutic or
therapeutically acceptable” refers to molecular entities and compositions that do not produce
15 an adverse, allergic, or other untoward reaction when administered to an animal, such as, for example, a human, as appropriate. The preparation of a therapeutic composition that contains at least one modified CGL enzyme isolated by the method disclosed herein, or additional active ingredient will be known to those of skill in the art, as exemplified by Remington’s
Pharmaceutical Sciences, 18th Ed., 1990, incorporated herein by reference. Moreover, for
20 animal (*e.g.*, human) administration, it will be understood that preparations should meet sterility, pyrogenicity, general safety, and purity standards as required by the FDA Office of Biological Standards.

[00130] As used herein, “pharmaceutically acceptable carrier” includes any and
all solvents, dispersion media, surfactants, antioxidants, preservatives (*e.g.*, antibacterial
25 agents, antifungal agents), isotonic agents, absorption delaying agents, salts, preservatives, drug stabilizers, gels, binders, excipients, such like materials and combinations thereof, as would be known to one of ordinary skill in the art (see, for example, Remington’s
Pharmaceutical Sciences, 18th Ed., 1990, incorporated herein by reference). Except insofar as
any conventional carrier is incompatible with the active ingredient, its use in the therapeutic
30 compositions is contemplated.

[00131] The compositions can be administered subcutaneously, intravenously, intraarterially, intraperitoneally, intramuscularly, by injection, by infusion, by continuous infusion, *via* a catheter, in lipid compositions (*e.g.*, liposomes), or by other methods or any combination of the foregoing as would be known to one of ordinary skill in the art (see, for example, Remington's Pharmaceutical Sciences, 18th Ed., 1990, incorporated herein by reference).

[00132] The modified polypeptides may be formulated into a composition in a free base, neutral, or salt form. Therapeutically acceptable salts include the acid addition salts, *e.g.*, those formed with the free amino groups of a proteinaceous composition, or which are formed with inorganic acids, such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, or mandelic acid. Salts formed with the free carboxyl groups can also be derived from inorganic bases, such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides; or such organic bases as isopropylamine, trimethylamine, histidine, or procaine. Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations may be administered in a variety of dosage forms, such as being formulated for parenteral administrations, such as injectable solutions, or aerosols for delivery to the lungs, or formulated for alimentary administrations, such as drug release capsules and the like.

[00133] The disclosed compositions suitable for administration may be provided in a pharmaceutically acceptable carrier with or without an inert diluent. Except insofar as any conventional media, agent, diluent, or carrier is detrimental to the recipient or to the therapeutic effectiveness of the composition contained therein, its use in administrable composition for use in practicing the methods is appropriate. Examples of carriers or diluents include fats, oils, water, saline solutions, lipids, liposomes, and the like, or combinations thereof. The composition may also comprise various antioxidants to retard oxidation of one or more component. Additionally, the prevention of the action of microorganisms can be brought about by preservatives, such as various antibacterial and antifungal agents, including but not limited to parabens (*e.g.*, methylparabens, propylparabens), chlorobutanol, phenol, sorbic acid, thimerosal or combinations thereof.

[00134] The disclosed compositions can be combined with the carrier in any convenient and practical manner, *i.e.*, by solution, suspension, emulsification, admixture, encapsulation, absorption, and the like.

[00135] The actual dosage amount of a composition administered to an animal patient can be determined by physical and physiological factors, such as body weight, severity of condition, the type of disease being treated, previous or concurrent therapeutic interventions, idiopathy of the patient, and on the route of administration. Depending upon the dosage and the route of administration, the number of administrations of a preferred dosage and/or a therapeutically effective amount may vary according to the response of the subject. The practitioner responsible for administration will, in any event, determine the concentration of active ingredient(s) in a composition and appropriate dose(s) for the individual subject.

[00136] Therapeutic compositions may comprise, for example, at least about 0.1% of an active compound. An active compound may comprise between about 2% to about 75% of the weight of the unit, or between about 25% to about 60%, for example, and any range derivable therein. Naturally, the amount of active compound(s) in each therapeutically useful composition may be prepared in such a way that a suitable dosage will be obtained in any given unit dose of the compound. Factors, such as solubility, bioavailability, biological half-life, route of administration, product shelf life, as well as other pharmacological considerations, will be contemplated by one skilled in the art of preparing such therapeutic formulations, and as such, a variety of dosages and treatment regimens may be desirable.

[00137] A dose may also comprise from about 500 microgram/kg body weight, about 1 milligram/kg body weight, about 5 milligram/kg body weight, about 10 milligram/kg body weight, about 50 milligram/kg body weight, about 100 milligram/kg body weight, about 750 milligrams/kg body weight or more per administration, and any range derivable therein. If the dose is administered weekly, the dose could be in the amount of 5 mg/kg body weight, or for example 350 mg of protein for a 70 kg subject.

XI. Combination Treatments

[00138] The compositions and methods provided herein may involve administration of a modified CGL enzyme in combination with a second or additional

therapy. Such therapy can be applied in the treatment of any disease that is associated with cyst(e)ine dependency. For example, the disease may be cystinuria.

[00139] Combination therapies may enhance the therapeutic or protective effect, and/or increase the therapeutic effect of another therapy. Therapeutic and prophylactic methods and compositions can be provided in a combined amount effective to achieve the desired effect, such as the elimination of cystine stones in the urinary tract or prevention of the formation of cystine stones in the urinary tract, kidney, and/or bladder. This process may involve administering both a modified CGL enzyme and a second therapy. A tissue, organ, or cell can be exposed to one or more compositions or pharmacological formulation(s) comprising one or more of the agents (*i.e.*, a modified CGL enzyme or a second agent), or by contacting the tissue, organ, and/or cell with two or more distinct compositions or formulations, wherein one composition provides 1) a modified CGL enzyme, 2) a second agent, or 3) both a modified CGL enzyme and a second agent. A combination therapy may be used in conjunction with shock wave therapy or surgical therapy.

[00140] The terms “contacted” and “exposed,” when applied to a cell, are used herein to describe the process by which a therapeutic construct is delivered to a target organ or are placed in direct juxtaposition with the target cell. To achieve stone dissolution, for example, both agents are delivered to a cell in a combined amount effective to dissolve the stone or prevent it from forming or reforming.

[00141] A modified CGL enzyme may be administered before, during, after, or in various combinations relative to a second cystinuria treatment. The administrations may be in intervals ranging from concurrently to minutes to days to weeks. When the modified CGL enzyme is provided to a patient separately from a second cystinuria treatment, one would generally ensure that a significant period of time did not expire between the time of each delivery, such that the two treatments would still be able to exert an advantageously combined effect on the patient. In such instances, one may provide a patient with the modified CGL enzyme and the second cystinuria therapy within about 12 to 24 h or 72 h of each other or within about 6-12 h of each other. In some situations, it may be desirable to extend the time period for treatment where several days (2, 3, 4, 5, 6, or 7) to several weeks (1, 2, 3, 4, 5, 6, 7, or 8) lapse between respective administrations.

[00142] It is contemplated that the modified CGL may be given on any day of day 1 to day 90 (this such range includes intervening days) or any combination thereof, and another treatment may be given on any day of day 1 to day 90 (this such range includes intervening days) or any combination thereof. Within a single day (24-hour period), the patient may be given one or multiple administrations of the treatment(s). After a course of treatment, there may be a period of time at which no treatment is administered. This time period may last 1-7 days, and/or 1-5 weeks, and/or 1-12 months or more (this such range includes intervening days), depending on the condition of the patient, such as their prognosis, strength, health, *etc.* Treatment cycles can be repeated as necessary.

[00143] Various combinations may be employed. For the example below a modified CGL enzyme is “A” and a second cystinuria therapy is “B”:

A/B/A B/A/B B/B/A A/A/B A/B/B B/A/A A/B/B/B B/A/B/B
 B/B/B/A B/B/A/B A/A/B/B A/B/A/B A/B/B/A B/B/A/A
 B/A/B/A B/A/A/B A/A/A/B B/A/A/A A/B/A/A A/A/B/A

[00144] Administration of any compound or therapy to a patient will follow general protocols for the administration of such compounds, taking into account the toxicity, if any, of the therapy. Therefore, there may be a step of monitoring toxicity that is attributable to the therapy.

A. Surgery

[00145] One of the most common methods for the removal of cystine stones is percutaneous nephrolithotomy, in which a keyhole incision is made in the back and a nephroscope is used to break up and remove the stones. Although this procedure is less invasive than open surgery, regular or spinal anesthesia is normally required along with a hospital stay of 2 to 3 days and a recovery time of a few weeks.

B. Shock wave therapy

[00146] Cystinuric patients often have recurrent episodes of stone formation and surgeries in their lifetime. Shock wave lithotripsy, the use of high-energy shock waves for stone fragmentation, can be used for treatment of cystine stones that are smaller than 1.5 cm. Cystine stones are the sturdiest of all urinary stones and lithotripsy is generally

ineffective in breaking them up. However, smaller cystine stones may be fragmented with lithotripsy because more frequent shocks at higher energy can be used.

C. Drug therapy

[00147] Drug therapy for cystine stone related medical conditions involves the use of thiol-containing drugs, such as D-penicillamine, α -mercaptopropionylglycine or Tiopronin (Thiola®), and captopril, to break the cystine disulfide bond and form more soluble mixed disulfides or cysteine itself. However, these drugs frequently give the patient various unpleasant side effects, such as gastrointestinal intolerance, rash and joint pain (Sakhaee and Sutton, 1996).

D. Other methods

[00148] Additional courses of treatment for removing cystine stones usually involve management of urinary cystine levels to reduce the risk of stone formation. These management methods include substantially increasing the intake of water (thereby increasing the urine volume and the amount of cystine that can be solubilized), dietary restrictions of sodium and methionine, which is a metabolic precursor of cystine, and oral administration of potassium citrate to increase the pH of the urine, thereby increasing the solubility of cystine.

[00149] An additional method for the treatment of cystine stones, which is a non-surgical and minimally invasive route, involves the delivery of chemical solutions to the kidneys via a nephrostomy catheter for the chemical dissolution of the stones, also known as chemodissolution. A variety of chemolytic agents have been used in this technique including sodium bicarbonate and the organic buffer tris-hydroxymethylene-aminomethane (tromethamine-E) at pH 10, both which act to provide a strongly alkaline environment to dissolve the cystine stones. Acetylcysteine is also frequently used in chemodissolution and dissolves the stones in a manner similar to D-penicillamine and Thiola® by breaking the cystine disulfide and forming more soluble disulfides. However, this dissolution method has a limited role in the treatment of cystine stones because these chemolytic agents perform slowly and can typically take weeks to months to dissolve stones (Ng and Stroom, 2001).

XII. Kits

[00150] Provided are kits, such as therapeutic kits. For example, a kit may comprise one or more therapeutic composition as described herein and optionally instructions

for their use. Kits may also comprise one or more devices for accomplishing administration of such compositions. For example, a subject kit may comprise a therapeutic composition and catheter for accomplishing direct intravenous injection of the composition into a target tissue. A kit may comprise pre-filled ampoules of a modified CGL enzyme, optionally formulated as a therapeutic composition, or lyophilized, for use with a delivery device.

[00151] Kits may comprise a container with a label. Suitable containers include, for example, bottles, vials, and test tubes. The containers may be formed from a variety of materials, such as glass or plastic. The container may hold a composition that includes a modified CGL enzyme that is effective for therapeutic or non-therapeutic applications, such as described above. The label on the container may indicate that the composition is used for a specific therapy or non-therapeutic application, and may also indicate directions for either *in vivo* or *in vitro* use, such as those described above. Kits will typically comprise the container described above and one or more other containers comprising materials desirable from a commercial and user standpoint, including buffers, diluents, filters, needles, syringes, and package inserts with instructions for use.

XIII. Examples

[00152] The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

Example 1 – 96-well plate screen for cyst(e)inease activity

[00153] Cysteinase enzymes degrade L-cysteine to pyruvate, ammonia, and H₂S, and convert L-cystine to pyruvate, ammonia, and thiocysteine ($k_{cat}/K_M \sim 0.2 \text{ mM}^{-1}\text{s}^{-1}$ and $0.5 \text{ mM}^{-1}\text{s}^{-1}$ respectively) (thiocysteine is further nonenzymatically degraded to L-cysteine and H₂S). A colorimetric assay for the detection of pyruvate using 3-methyl-2-benzothiazolinone hydrazone (MBTH) (Takakura et al., 2004) was scaled to a 96-well plate format for screening small libraries and for ranking clones with the greatest cystine and/or

cysteine lyase activity. This plate screen provides a facile method for picking the most active clones from the mutagenic libraries.

[00154] Single colonies containing mutagenized hCGL, or hCGL controls were picked into 96-well culture plates containing 100 μ L of TB media/well containing 50 μ g/mL kanamycin. These cultures were then grown at 37°C on a plate shaker for 12 h. After cooling to 25°C, an additional 100 μ L of media/well containing 50 μ g/mL kanamycin and 1 mM IPTG was added. Expression was performed at 25°C with shaking for 16 h, following which 100 μ L of culture/well was transferred to a 96 well assay plate. The assay plates were then centrifuged to pellet the cells, the media was removed, and the cells were lysed by addition of 100 μ L/well of B-PER protein extraction reagent (Pierce). After clearing the lysate by centrifugation, 90 μ L of supernatant was transferred to each well of a His-Sorb plate (Qiagen) and incubated at 10°C with slow agitation for three hours. The resulting supernatant was then discarded, and the plate was washed for a total of three times by adding 200 μ L PBS to each well, agitating the plate slowly at 4°C for 15 minutes, and aspirating off the wash buffer. Then, 100 μ L reaction buffer containing 0.5 mM cystine was added to each well of the His-Sorb plate and incubated at 37°C for two hours. Following which 50 μ L of the reaction were transferred to a separate 96 well plate and derivatized by addition of 150 μ L of MBTH working solution/well. The plates were then heated at 50°C for 40 min and after cooling were read at $\lambda = 320$ nm in a microtiter plate reader. Clones displaying greater activity than parental controls were selected for further characterization.

Example 2 – Construction of mutagenized libraries of engineered human cysteinase

[00155] Structural and phylogenetic analyses suggested that residues A51, E59, L91, T163, V166, T189, P193, A200, N234, T311, E339, I353, K361 and N362 could play a role in cysteinase-mediated cystine degradation. Various degenerate codon saturation mutagenesis libraries were constructed at these positions by overlap extension PCR. The resulting PCR products were digested with NcoI and EcoRI and ligated into a pET28a vector with T4 DNA ligase. The ligations were transformed directly into *E. coli* (BL21-DE3) and plated on LB-kanamycin plates for use in subsequent screening experiments as described in Example 1. All libraries were screened in two-fold excess of their theoretical size. Clones displaying enhanced activity compared to parental control reactions were isolated and the sequences were determined to identify the mutations.

Example 3 – Expression and purification of cysteinase variants

[00156] Cysteinase variants displaying enhanced activity in the 96-well plate screens were cultured in Terrific Broth (TB) media containing 50 µg/mL kanamycin in shaker flasks at 250 rpm until reaching an OD₆₀₀ of 0.5-0.6. At this point, the cultures were
5 switched to a shaker at 25°C and induced with 0.5 mM IPTG and allowed to express protein for an additional 12 h. Cell pellets were then collected by centrifugation and re-suspended in an IMAC buffer (10 mM NaPO₄ /10 mM imidazole / 300 mM NaCl, pH 8). After lysis by a French pressure cell, lysates were centrifuged at 20,000 x g for 20 min at 4°C, and the resulting supernatant applied to a nickel IMAC column, washed with 10-20 column volumes
10 of IMAC buffer, and then eluted with an IMAC elution buffer (50 mM NaPO₄ / 250 mM imidazole / 300 mM NaCl, pH 8). Fractions containing enzyme were then incubated with 5 mM pyridoxal phosphate (PLP) for an hour at 25°C. Using a 10,000 MWCO centrifugal filter device (Amicon), proteins were buffer exchanged several times into a 100 mM PBS, 10% glycerol, pH 7.3 solution. Aliquots of cysteinase-variant enzymes were either analyzed
15 immediately for their biophysical properties or flash frozen in liquid nitrogen and stored at -80°C. The hCGL-variant enzymes purified in this manner were >95% homogeneous as assessed by SDS-PAGE and coomassie staining.

Example 4 – Coupled lactate dehydrogenase assay for determining kinetics

[00157] For determining the steady-state kinetic parameters of cysteinase
20 variants, a coupled, continuous spectrophotometric assay was developed. This assay relies on the utilization of pyruvate, produced by the cysteinase catalyzed reaction with cystine, by an auxiliary enzyme namely lactate dehydrogenase (LDH). LDH oxidizes NADH in the presence of pyruvate stoichiometrically generating lactate and NAD⁺. The oxidation of NADH is proportional to the pyruvate and this, in turn, is proportional to the activity of the
25 cysteinase enzymes. The disappearance of NADH was monitored spectrophotometrically at 340 nm as a function of time using a wide range of cystine concentrations and the linear part of the traces were used to calculate reaction rates before the depletion of 10% of the substrate (conditions of initial velocity). The substrate concentration-dependent observed rates were normalized to the enzyme concentration used for the reaction and subsequently were plotted
30 as a function of substrate concentration. The resulting data were fit to the Michaelis-Menten equation for the calculation of the steady-state parameters. The conversion of the NADH absorbance to absolute concentrations was calculated using the molar absorption extinction

coefficient for NADH ($6.22 \text{ mM}^{-1} \text{ cm}^{-1}$). The variants were tested using this assay to determine steady state kinetic parameters and the resulting k_{cat}/K_M values were directly compared to those of a reference cysteinase variant (hCGL-H55E-E59T-T336D-E339V; see U.S. Appln. No. 15/977,246, which is incorporated by reference herein in its entirety) assayed in parallel. Numerous variants displayed increases in k_{cat}/K_M for degrading cystine (Table 1, fold improvement for k_{cat}/K_M).

Table 1. Fold improvement of k_{cat}/K_M for cystine degradation relative to a reference cysteinase (hCGL-H55E-E59T-T336D-E339V).

SEQ ID NO:	Mutations	Fold improvement k_{cat}/K_M
6	P193A, T311G, E339V, I353S	2.7
7	A200P, T311G, E339V, I353S	2.5
8	P193A, A200P, T311G, E339V, I353S	2
9	H55E, E59T, L91M, N234K, T336D, E339V	1.3
10	H55E, E59T, L91S, N234K, T336D, E339V	1.4
11	T189S, P193G, T311G, E339V, I353S	1.3
12	T163R, T311G, E339V, I353S	1.4
13	A51W, H55E, E59T, T336D, E339V	1.8
14	H55E, P193A, T311G, T336D, E339V, I353S	1.5
15	A200H, T311G, E339V, I353S	1.6
16	T311G, E339V, I353S	2.6
17	E59I, E339V, I353S	1.5
18	L91M, E339V	1.3
19	E339V, I353S	2.9
20	E339V	2.8
nd = not determined		

[00158] All of the methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

REFERENCES

The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference.

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WHAT IS CLAIMED IS:

1. An isolated, modified primate cystathionine- γ -lyase (CGL) enzyme comprising the following substitutions relative to a native human CGL amino acid sequence (SEQ ID NO: 1), wherein the modified enzyme has both cystinase and cysteinase activity, said substitutions being: alanine at position 193, glycine at position 311, valine at position 339, and serine at position 353.
2. The isolated, modified CGL enzyme of claim 1, wherein the modified CGL enzyme is a modified human CGL enzyme.
3. The isolated, modified CGL enzyme of claim 1 or 2, wherein the modified CGL enzyme is at least 95% identical to SEQ ID NO: 1.
4. The isolated, modified CGL enzyme of any one of claims 1-3, wherein the modified CGL has the sequence of SEQ ID NO: 6.
5. The isolated, modified CGL enzyme of any one of claims 1-4, wherein the modified CGL enzyme has a k_{cat}/K_M for L-cystine and/or L-cysteine that is greater than the k_{cat}/K_M of human cystathionine- γ -lyase.
6. The isolated, modified CGL enzyme of any one of claims 1-5, wherein the modified CGL enzyme has a k_{cat}/K_M for L-cystine that is greater than $10,000 \text{ s}^{-1}\text{M}^{-1}$ and/or wherein the modified CGL enzyme has a k_{cat}/K_M for L-cysteine that is greater than $1,000 \text{ s}^{-1}\text{M}^{-1}$.
7. The isolated, modified CGL enzyme of any one of claims 1-6, further comprising a heterologous peptide segment or a polysaccharide.
8. The isolated, modified CGL enzyme of claim 7, wherein the heterologous peptide segment is an XTEN peptide, an IgG Fc, an albumin, or an albumin binding peptide.
9. The isolated, modified CGL enzyme of claim 7, wherein the polysaccharide comprises polysialic acid polymers.
10. The isolated, modified CGL enzyme of any one of claims 1-9, wherein the enzyme is coupled to polyethylene glycol (PEG).

11. The isolated, modified CGL enzyme of claim 10, wherein the enzyme is coupled to the PEG via one or more lysine residues.
12. A nucleic acid comprising a nucleotide sequence encoding the enzyme of any one of claims 1-6.
- 5 13. The nucleic acid of claim 12, wherein the nucleic acid is codon optimized for expression in bacteria, fungus, insects, or mammals.
14. The nucleic acid of claim 13, wherein the bacteria are *E. coli*.
15. The nucleic acid of claim 14, wherein the nucleic acid comprises a sequence according to SEQ ID NOs: 96.
- 10 16. An expression vector comprising the nucleic acid of any one of claims 12-15.
17. A host cell comprising the nucleic acid of any one of claims 12-15.
18. The host cell of claim 17, wherein the host cell is a bacterial cell, a fungal cell, an insect cell, or a mammalian cell.
19. A therapeutic formulation comprising an enzyme of any one of claims 1-10, or the
15 nucleic acid of any one of claims 12-15, in a pharmaceutically acceptable carrier.
20. Use of a formulation of claim 19 in the manufacture of a medicament for treating a subject having or at risk of developing cystinuria.
21. The use of claim 20, wherein the subject is maintained on a L-cystine and/or L-cysteine restricted diet.
- 20 22. The use of claim 20, wherein the subject is maintained on a methionine-restricted diet.
23. The use of claim 20, wherein the subject is maintained on a normal diet.
24. The use of claim 20, wherein the subject is a human patient.
25. The use of claim 20, wherein the formulation is to be administered intravenously, intraarterially, intraperitoneally, intramuscularly, intravascularly, subcutaneously, by
25 injection, by infusion, by continuous infusion, or via a catheter.

26. The use of claim 20, wherein the subject has previously been treated for cystinuria and the enzyme is to be administered to prevent the recurrence of cystinuria.
27. The use of claim 20, further comprising at least a second cystinuria therapy to be administered to the subject.
- 5 28. The use of claim 27, wherein the second cystinuria therapy is a surgical therapy or a shock wave therapy.
29. The use of claim 27, wherein the second cystinuria therapy comprises an agent or drug selected from the group consisting of: bicarbonate, tris-hydroxymethylene-aminomethane (tromethamine-E), acetylcysteine, D-penicillamine, α -
10 mercaptopropionylglycine, and captopril.
30. The use of claim 20, wherein the medicament reduces the level of cysteine and/or cystine in the subject's plasma and/or serum.
31. The use of claim 20, wherein the medicament reduces the level of cysteine and/or cystine in the subject's urine.
- 15 32. The use of claim 20, wherein the medicament prevents the formation of cystine stones in the subject's urinary tract.
33. The use of claim 20, wherein the medicament prevents the formation of cystine stones in the subject's kidneys.
34. The use of claim 20, wherein the medicament reduces the number of cystine stones in
20 the subject's kidneys.
35. Use of an isolated or modified CGL enzyme of any of claims 1 to 11 or a composition comprising said modified CGL enzyme in the manufacture of a medicament for therapeutic application to a cystinuria patient.