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Expression, Purification, and In Vitro Cysteine-10 Modification of Native Sequence Recombinant Human Transthyretin

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Abstract

Transthyretin (TTR) is a serum protein that is also a prominent component of deposits in two different types of systemic amyloid disease, senile systemic and familial TTR amyloidoses. Studies of recombinant TTR (rTTR) have provided many insights into the relationship between protein structure and amyloidogenicity. Yet, there is no existing recombinant system that results in high yield production of a protein that is identical in primary structure to human TTR. To date, most published studies have generated rTTR using the human gene sequence, which is poorly expressed in *E. coli*. In addition, the gene sequence has been flanked by a 3' AUG start codon to initiate translation, resulting in the expression of a protein containing an N-terminal methionine residue not present in the human protein. We present an improved technique which can be used to generate large quantities of human native sequence TTR. Our recombinant system utilizes a gene containing codons altered for efficient expression in *E. coli* and an N-terminal polyhistidine tag for simplified purification. Optimization of this system was accomplished by generating a modified polyhistidine tag that was efficiently removed by dipeptidyl aminopeptidase I (DAPase). This is the first report detailing an effective and useful method for producing rTTR containing an amino acid sequence identical to human TTR. Furthermore, we describe the thiol modification of the recombinant protein to achieve exact replication of the several prominent post-translationally modified forms of TTR that have been identified in human serum.

Introduction

Transthyretin (TTR) is a 127-amino acid (M_r human TTR, 13761 Da) serum protein that forms homo-tetramers under physiological conditions. In circulation, TTR binds and transports thyroxine as well as vitamin A through an association with holo-retinol binding protein. In addition to its normal biological role, TTR can aggregate and has been implicated in several pathologies. In familial TTR amyloidosis (ATTR), more than 80 single point mutations in the coding region sequences of the TTR gene can give rise to variant proteins that are prone to forming amyloid deposits in various organs [1,2]. ATTR is often clinically sub-categorized as

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either familial amyloidotic polyneuropathy (FAP) or familial amyloidotic cardiomyopathy (FAC) depending on organ involvement. In senile systemic amyloidosis (SSA), the amyloid deposits consist of wild type TTR [3] and the most prevalent clinical symptom is cardiomyopathy.

ATTR-related amino acid substitutions have been shown to destabilize TTR *in vitro* leading to the formation of amyloid fibrils [4]. However, wild type TTR is also known to form amyloid fibrils, as is the case in SSA, despite the lack of destabilizing mutations. Yet, at the post-translational level, wild type transthyretin is heterogeneous, *i.e.*, the single cysteine residue in each monomer (Cys10) readily forms disulfide bonds with a variety of thiol reactive molecules. In fact, only a small percentage (10-15%) of TTR in circulation contains reduced Cys10, while the majority of the protein is either *S*-sulfonated or *S*-cysteinylated [5-8]. As thiol composition of many proteins including TTR may be dependent on age [9,10], we have hypothesized that age-associated changes in the distribution of TTR mixed disulfides may be relevant in the onset of SSA and the penetrance of ATTR.

Investigating the pathologic mechanism and cellular effects of TTR-associated forms of amyloidosis requires the use of relatively large quantities of protein. It is also critical to generate proteins which contain amino acid sequences and chemical modifications identical to those identified in clinical samples. The purification of TTR from pooled human serum is problematic due to the limiting volume of clinical specimens. In addition, there is a high level of TTR structural heterogeneity resulting from genetic polymorphisms and post-translational modification of the protein. We sought to generate a high yield source of recombinant human TTR that contained the exact native protein sequence, *i.e.* TTR devoid of additional or altered amino acids resulting from the design of the expression vector (N-terminal methionine or affinity tags). To accomplish this, we adapted an existing protocol for expressing polyhistidine-tagged human transthyretin with altered codon usage for expression in *E. coli* [11], and then using site-directed mutagenesis, modified the polyhistidine tag to optimize its removal by dipeptidyl aminopeptidase I (DAPase). The homogeneous product (recombinant TTR, rTTR) was chemically treated to create *S*-sulfonated or *S*-cysteinylated TTR, both prominent species identified in human plasma. These *in vitro* modified rTTR proteins should prove useful in examining the role of Cys10 disulfide status in the TTR-related amyloidoses.

Materials and Methods

Construction of expression vector

A synthetic human TTR gene containing altered codon usage for expression in *E. coli* was constructed and cloned into the pQE30 plasmid (Qiagen) as described by Matsubara et al. [11]. The resulting rTTR incorporated a polyhistidine tag of the following amino acid sequence: MRGSHHHHHHGS.

Modification of polyhistidine tag

The cDNA region encoding the N-terminal polyhistidine tag of the rTTR was modified in two consecutive steps by site-directed mutagenesis (QuikChange XL Site-Directed Mutagenesis Kit, Stratagene). The sequences of the forward mutagenic primers were as follows, with the bolded and underlined letters indicating the mutation sites and ^ indicating the deletion position (reverse primers were the reverse complement of the forward primers): Step 1: 5'-CGCATCACCATCACCATCACGGAC**AC**GGTCCGACCGGTACCGG-3' Step 2: 5'-GGAGGAGAAATTAAC**TATGA****AA**[^]CATCACCATCACCATCACGG-3' The amino acid changes resulting from these mutations were Arg2Lys, ΔGly3, ΔSer4 and Ser12His. The final polyhistidine tag sequence was MKHHHHHHGH. Direct DNA sequencing of the complete

coding region in the expression plasmid was performed to verify that the correct mutations were present. The expressed protein was examined by nanospray mass spectrometry.

Expression of polyhistidine-tagged protein

The rTTR-pQE30 expression plasmid was introduced into M15 [pREP4] cells by heat shock and the cells were plated onto Luria broth (LB)-agar media containing 25 µg/ml kanamycin and 100 µg/ml ampicillin and incubated overnight at 37 °C. A single colony was isolated and cultured overnight at 37 °C in LB/kan/amp (LB media containing 25 µg/ml kanamycin and 100 µg/ml ampicillin). This culture was used to inoculate 500 ml of LB/kan/amp, which was then grown at 37 °C until OD₆₀₀ = 0.6. Protein expression was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) at 1 mM and incubating for 5 h at 37 °C. The cells (typically 2-3 g wet weight per 500 ml culture) were harvested by centrifugation, resuspended in cell lysis buffer (50 mM sodium phosphate, 300 mM sodium chloride, 10 mM imidazole, pH 8.0 with or without 20 mM 2-mercaptoethanol) at 3 ml per gram wet weight cells, supplemented with hen egg white lysozyme at 1 mg/ml, and incubated for 30 min at 4 °C. The cells were lysed by sonication at 4 °C and the supernatant was clarified by centrifugation and passage through 0.22 µm syringe tip filters.

Purification of polyhistidine-tagged protein

The clarified cell lysate was loaded onto a 3-ml nickel-nitrilotriacetic acid (Ni-NTA) agarose column (Qiagen) and rinsed with wash buffer (50 mM sodium phosphate, 300 mM sodium chloride, 20 mM imidazole, pH 8.0 with or without 20 mM 2-mercaptoethanol) until OD₂₈₀ < 0.01. The polyhistidine-tagged rTTR was eluted from the column with elution buffer (50 mM sodium phosphate, 300 mM sodium chloride, 250 mM imidazole, pH 8.0 with or without 20 mM 2-mercaptoethanol) and fractions of OD₂₈₀ > 0.05 were collected and pooled. The pooled fractions were then exhaustively dialyzed against TAGzyme buffer (150 mM sodium chloride, 20 mM phosphate, pH 7.0) using a 7000 MWCO Slide-A-Lyzer cassette (Pierce).

Removal of polyhistidine tag and purification of protein

Dipeptidyl aminopeptidase I (DAPase) was activated by incubating the desired quantity (50 mU enzyme per mg polyhistidine-tagged rTTR) for 5 min at room temperature with an equal volume of either 20 mM cysteamine or 20 mM dithiothreitol (DTT). The activated DAPase was then added to the polyhistidine-tagged rTTR solution and incubated for 48 h at 37 °C. The reaction mixture was loaded onto a 0.5-ml Ni-NTA agarose column, which was then flushed with wash buffer. Fractions of OD₂₈₀ > 0.05 were collected, pooled, dialyzed against 100 mM potassium chloride, 10 mM Tris, pH 7.5 using a 7000 MWCO Slide-A-Lyzer cassette, and then stored at -80 °C.

Polyacrylamide gel electrophoresis

SDS-PAGE samples were dissolved in reducing sample buffer (10 mM Tris, 1 mM EDTA, 2.5% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 0.001% (w/v) bromphenol blue, pH 8.0) and heated in a boiling water bath for 5 min. Electrophoresis was conducted on 10-15% gradient PhastGels with SDS-PAGE buffer strips (GE Healthcare) using a PhastSystem electrophoresis unit (GE Healthcare) according to the manufacturer's directions. Total protein was visualized by Coomassie blue staining using the PhastSystem gel developer unit.

Cysteine 10 chemical modification

Purified rTTR was *S*-sulfonated or *S*-cysteinylation at Cys10 by exchanging the recombinant protein solution into filtered reaction buffer (100 mM potassium chloride, 1 mM EDTA, 10 mM potassium phosphate, pH 7.0) containing either 40 mM sodium tetrathionate for *S*-sulfonation [12,13] or 400 µM cystine for *S*-cysteinylation using an EconoPak 10-DG desalting

column (Bio-Rad). The solutions were incubated for 15 h at room temperature then analyzed by nanospray mass spectrometry to verify the modifications and to determine heterogeneity with respect to mass. Products of incomplete Cys10 modification were exchanged into fresh reaction buffer and incubated again for 15 h at room temperature. Products indicating 95-100% reaction completion were exchanged into 100 mM potassium chloride, 10 mM Tris, pH 7.5 and stored at -80 °C.

Nanospray mass spectrometry

Samples were prepared for mass spectrometric analysis by reversed phase high pressure liquid chromatography (RP-HPLC) using an Agilent 1100 series HPLC system. Approximately 5 µg of sample was applied to an analytical Zorbax Poroshell 300SB-C8 HPLC column (75 mm long, 2.1 mm ID, 5 µm particle size) pre-equilibrated in 95% buffer A (5% acetonitrile, 0.1% trifluoroacetic acid in water), 5% buffer B (0.085% trifluoroacetic acid in acetonitrile). The column was eluted by increasing the concentration of buffer B linearly to 85% over 5 min at 1 ml/min. The entire protein peak (determined online with an Agilent G1314A variable wavelength detector set to measure OD210) was collected, dried in a SPD111V centrifugal concentrator (ThermoSavant), and stored at -20 °C.

Samples were dissolved in electrospray buffer (50% acetonitrile, 0.1% formic acid in water) at approximately 0.5 g/l and introduced into a Micromass Quattro II triple quadrupole mass spectrometer by nanospray. Data were obtained by scanning over the range m/z 200-2000 and then deconvoluted using the MAXENT function of the MASSLYNX (V3.4) operating program. TTR species were assigned to the following observed masses: unmodified rTTR, 13762 (calc. M_r 13761); S-sulfonated rTTR, 13840 (calc. Δ +80); S-cysteinylation rTTR, 13880 (calc. Δ +119). The error associated with data collection and deconvolution was assumed to be ± 3 Da.

Circular dichroism (CD) spectroscopy

CD spectra of the unmodified, S-sulfonated, and S-cysteinylation rTTR in 10 mM sodium phosphate, 1 mM EDTA, pH 7.4 at a protein concentration of 6-8 µM were collected at 25 °C on an Aviv 215 spectropolarimeter (Aviv Biomedical) continuously purged by nitrogen gas and equipped with a thermoelectric temperature controller. Far UV spectral measurements (185 – 250 nm, 1 nm bandwidth) were determined in a 1.0 mm path length quartz cell. Spectra were recorded at 1 nm intervals with a typical averaging time of 15 – 30 s. CD data, smoothed by using the Aviv noise reduction routine, are presented in units of molar ellipticity per residue.

Results

MRGS(6H)GS-rTTR binds 2-mercaptoethanol in expression/purification buffers

Polyhistidine-tagged rTTR (MRGS(6H)GS-rTTR) was expressed and purified as described [11]. The resulting protein appeared as a single band on SDS-PAGE at a position consistent with the expected mass (data not shown). However, nanospray MS analysis of this product (Fig. 1A) revealed that the mass (M_r 15240) was 78 u higher than the expected mass (M_r 15162). A less abundant peak at M_r 15254 (representing an additional +14 u shift) was also observed. The presence of these unpredicted mass shifts was initially assumed to be the result of chemical adduction at the lone cysteine of TTR (Cys10). The validity of this assumption was tested by treating the rTTR sample with 10 mM DTT for 1 h and reanalyzing the product by MS. The resulting mass spectrum (Fig. 1B) indicated that the most abundant form displayed the mass expected for the polyhistidine-tagged rTTR (M_r 15162). However, a second species with a +15 u peak (M_r 15177) was also present. Since the neutral mass of 2-mercaptoethanol is 78.13 and this was the only thiol-reactive component present in the expression or purification buffers, we hypothesized that this molecule bound to MRGS(6H)GS-rTTR during the procedure.

Expression and purification was then repeated using buffers in which 2-mercaptoethanol was omitted, and the product was analyzed by nanospray MS. As indicated in Fig. 1C, the observed mass (M_r 15163) was consistent with the expected mass for MRGS(6H)GS-rTTR. The additional +14 u species has been identified as MRGS(6H)GS-rTTR containing N-methyl methionine [R. Théberge, to be published elsewhere].

MRGS(6H)GS-rTTR binds the DAPase activator cysteamine

Removal of the polyhistidine tag from the expressed protein was performed at 37 °C using dipeptidyl aminopeptidase I (DAPase) at an enzyme-to-substrate ratio equal to 100 mU per 1 mg of MRGS(6H)GS-rTTR. The enzyme manufacturer (Qiagen) recommends that DAPase be activated with an equal volume of 20 mM cysteamine prior to incubation with the polyhistidine-tagged substrate, and this procedure was included. Following digestion for 24 h, the DAPase, which itself is polyhistidine-tagged, was removed by Ni-NTA agarose chromatography. The purified product was then analyzed by nanospray MS. The resulting mass spectrum (Fig. 2A) exhibited four peaks; the lowest M_r component was observed to have a mass (M_r 13763) which corresponded, within experimental error, to the expected mass of rTTR. The peak at M_r 13907 was consistent with a species resulting from incomplete digestion of the polyhistidine tag in which the GS dipeptide linker was still attached to the N-terminus of the protein (GS-rTTR). Two additional peaks were also observed (M_r 13838 and M_r 13982) that were mass shifted by +75 u from the rTTR and GS-rTTR assigned peaks. Cysteamine has a neutral mass of 77.15 and is thiol-reactive. As 2-mercaptoethanol was already determined to bind Cys10, we hypothesized that rTTR formed a mixed-disulfide with cysteamine during the dipeptidase reaction. This possibility was examined by treating the Ni-NTA purified product with 10 mM DTT for 1 h and analyzing the resulting sample by nanospray MS. The mass spectrum (Fig. 2B) no longer showed the components with +75 u increments and thus provided supporting evidence for our hypothesis. In a subsequent trial digestion, DAPase was activated with an equal volume of 20 mM DTT instead of cysteamine. This reaction was carried out for a longer period of time (75 h) in an attempt to eliminate the partially digested species (GS-rTTR). Nanospray MS analysis of the product from this reaction (Fig. 2C) indicated that >95% of the sample had a mass (M_r 13762) that corresponded to native sequence TTR.

Optimization of polyhistidine tag for DAPase digestion

The digestion of MRGS(6H)GS-rTTR by DAPase at 100 mU enzyme per mg substrate was close to completion after 75 h at 37 °C (Fig. 2C). These conditions are sub-optimal with respect to the amount of enzyme required, as well as the length of time required for reaction incubation. We hypothesized that the presence of two GS dipeptides, which are poor substrates for DAPase [Qiagen data], limited the rate of digestion. These residues were present in the polyhistidine tag as a consequence of design for the BamHI restriction site on the pQE30 plasmid and had no function at the protein level. Site-directed mutagenesis was used to remove one of the GS pairs and to convert the other to GH, a dipeptide rapidly digested by DAPase [Qiagen data]. Furthermore, the replacement of the arginine residue at position 2 with lysine was included as part of the tag modification in order to limit the N-terminal methionine processing in *E. coli* [14]. The primary structure of the original and optimized polyhistidine tags are illustrated in Fig. 3A.

A pilot expression was conducted to test the optimized plasmid. Nanospray MS analysis of the Ni-NTA purified protein indicated a largely homogeneous species of M_r 15038, consistent with the expected mass of MK(6H)GH-rTTR. A minor species of +14 u was also detected and likely corresponds to MK(6H)GH-rTTR containing N-methylated methionine (Fig. 3B). This product was treated with DAPase at 50 mU per mg protein and incubated for 48 h at 37 °C. The digest was then purified on a Ni-NTA agarose column and analyzed by nanospray MS. A homogeneous species at a neutral mass (M_r 13761) was noted and is consistent with the

expected mass of rTTR (Fig. 3C). These results demonstrate the improved digestion kinetics of the optimized polyhistidine tag.

Several large scale (500 ml) cultures were prepared and analyzed at each stage of purification to fully characterize the efficiency and robustness of our optimized expression system. Typical expressions (see Fig. 4 and Table 1) yielded 40-50 mg of MK(6H)GH-rTTR with no more than 1-2% impurity as detected by SDS-PAGE or nanospray MS. Removal of the polyhistidine tag was accomplished with 50 mU DAPase per mg MK(6H)GH-rTTR and the reaction was completed typically after 24-48 h at 37 °C. Over 95% of the expressed protein was normally recovered after Ni-NTA agarose purification and product purity was determined to be >98% as assessed by SDS-PAGE and nanospray MS.

S-sulfonation and S-cysteinylation

We have hypothesized that age-associated changes in TTR thiol chemistry may contribute to the onset of ATTR and SSA by triggering amyloid formation through perturbation of native structure. We sought to generate rTTR species that were identical in structure to those found in serum to investigate this hypothesis. Purified rTTR was *S*-sulfonated using a published procedure [12]. The reaction product was characterized by nanospray MS (Fig. 5, left) and indicated a homogeneous species of M_r 13840, consistent with *S*-sulfonated TTR (rTTR-SO₃). A similar reaction was also conducted using a solution containing cystine instead of sodium tetrathionate. The product was characterized by nanospray MS (Fig. 5, right). A homogeneous species consistent with *S*-cysteinyllated TTR (rTTR-Cys) was detected (M_r 13879). Both the *S*-sulfonated and *S*-cysteinyllated forms of TTR were obtained at quantitative yields.

Far-UV CD spectra of unmodified, *S*-sulfonated, and *S*-cysteinyllated rTTR were separately collected in order to verify proper folding of the recombinant proteins and to investigate the effect of the Cys10 modifications on secondary structure. The shapes and amplitudes of the spectra are characteristic of proteins with a high content of β -sheet structure and consistent with spectra previously reported for TTR [15-17]. Though the spectra of the three TTR forms are similar, minor differences in amplitudes in the 205-225 nm and 190-200 nm wavelength ranges may reflect slight reorganizations in protein structure as a result of the post-translational modifications.

Discussion

In this report, we detail the development and characterization of a high yield expression system for generating native sequence recombinant human TTR and several chemically modified variants. The procedure for generating polyhistidine-tagged TTR was adapted from an existing protocol [11], then optimized and extended to incorporate proteolytic removal of the entire tag.

Using the original method of Matsubara et al., we obtained polyhistidine-tagged rTTR (MRGS(6H)GS-rTTR) that was covalently bound to 2-mercaptoethanol, a component of the cell lysis and Ni-NTA agarose buffers. It is possible that this adduct could alter the physicochemical properties of the recombinant protein and influence downstream analyses. Furthermore, to more accurately model the properties of human TTR, it is desirable to remove the polyhistidine tag prior to experimentation since it can alter protein structure and influence aggregation properties, particularly when divalent cations are present. Biophysical studies using 2-mercaptoethanol-bound and polyhistidine-tagged rTTR [11,18,19] may yield results that deviate significantly from identical studies conducted on native sequence rTTR containing a free thiol group. In addition, our results suggest that mass spectrometric analysis of any expression product is essential when using reductants to optimize the yield of recombinant

systems. More routine analyses such as non-reducing SDS-PAGE do not provide the mass resolution necessary to detect small molecule chemical adducts.

We determined that the 2-mercaptoethanol adduct could be removed by treatment with DTT. More simply, the correct product was obtained by eliminating 2-mercaptoethanol from all buffers. We then sought to remove the polyhistidine tag using DAPase, a protease that cleaves consecutive N-terminal dipeptides until one of five stop sequences is reached. One of these motifs (Xaa-Pro) describes the N-terminus of human TTR, making it an ideal enzyme for this application. However, we determined that high concentrations (100 mU DAPase per mg MRGS (6H)GS-rTTR) and long incubation periods (75 h) were necessary to completely digest the tag. To overcome this problem, we used site-directed mutagenesis to create a tag sequence that was more amenable to DAPase digestion. The optimized product, MK(6H)GH-rTTR, was a much more efficient substrate for DAPase (50 mU enzyme per mg MK(6H)GH-rTTR, <48 h digestion) and yielded a homogeneous product with a measured mass (M_r 13762) that was consistent with human TTR. One further procedural modification was necessary to generate the correct product. We found that the thiol-reactive activator of DAPase, cysteamine, covalently bound rTTR during the digestion reaction. We determined that DTT could be substituted as an activator in order to eliminate this byproduct.

Using the optimized expression plasmid, we routinely obtained 40-50 mg of pure MK(6H)GH-rTTR per 500 ml culture. The tag was digested with DAPase and rTTR was obtained at near quantitative yield. To our knowledge, this procedure is the first to yield rTTR with a primary structure that is completely identical to human TTR, *i.e.* no affinity tags or N-terminal methionine are present on the final recombinant protein. This product was then chemically modified by *S*-sulfonation and *S*-cysteinylation reactions to replicate the most abundant TTR forms present in human serum [5-8]. Mass spectrometric analyses verified the chemical derivatizations and provided an assessment of the homogeneity of the products.

Separate CD spectroscopic analyses of the unmodified, *S*-sulfonated, and *S*-cysteinylation forms of rTTR indicated that each had a high degree of β -sheet secondary structure. In general, the far UV CD spectra were similar to data reported for other rTTR products [15-17], suggesting proper folding of our proteins. In addition, gel filtration HPLC verified that each species formed tetramers in physiological buffers, again supporting proper folding (data not shown). Minor differences between the unmodified and mixed disulfide forms in the 205-225 nm and 190-200 nm wavelength ranges might indicate structural perturbation by the modifications, consistent with our hypothesis. However, determination of the precise nature of these differences requires more rigorous thermodynamic and kinetic analyses.

In summary, this report details the production of native sequence recombinant human TTR and the Cys10 modification of this product by *S*-sulfonation and *S*-cysteinylation. The chemical structure, folding, and self-association of these recombinant proteins were characterized by mass spectrometry, CD spectroscopy, and gel filtration HPLC and were determined to be consistent with respect to human TTR. It is anticipated that future studies with these proteins will help elucidate the role of Cys10 in the pathological aggregation of wild type TTR.

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Abbreviations

TTR

	transthyretin
rTTR	recombinant human transthyretin
MS	mass spectrometry
DTT	dithiothreitol

Fig. 1.

Polyhistidine-tagged rTTR binds 2-mercaptoethanol. MRGS(6H)GS-rTTR was expressed and purified by the method of Matsubara et al. [11], then analyzed by nanospray MS (A). A portion of the product was treated with DTT and analyzed in the same way (B). A separate expression and purification was also conducted using the identical procedure except that 2-mercaptoethanol was not included in the cell lysis buffer or the Ni-NTA wash and elution buffers. The product was then analyzed by nanospray MS (C).

Fig. 2. rTTR binds the DAPase activator cysteamine. Nanospray MS was used to analyze the product of the DAPase digestion of MRGS(6H)GS-rTTR at 100 mU enzyme per mg of substrate. Analysis was conducted on a 24 h digestion after activating DAPase with 1:1 (v:v) 20 mM cysteamine (A) as indicated by the supplier. A portion of this product was treated with DTT and subsequently analyzed (B). A separate 75 h digestion was conducted after activating DAPase with 1:1 (v:v) 20 mM DTT and the product was analyzed in the same way (C).

Fig. 3. Optimization of the polyhistidine tag. Site-directed mutagenesis was used to remove kinetically unfavorable dipeptides from the original polyhistidine tag (A, top). The optimized sequence (A, bottom) also contains an arginine to lysine substitution to minimize N-terminal methionine processing by *E. coli*. The new polyhistidine-tagged rTTR was then expressed, purified, and analyzed by nanospray MS (B). This product was digested with DTT-activated DAPase at 50 mU enzyme per mg substrate for 48 h and analyzed by nanospray MS (C).

Fig. 4. SDS-PAGE analysis of rTTR at different stages of purification. Lane 1, clarified cell lysate (2 μ g), Lane 2, Ni-NTA purified MK(6H)GH-rTTR (0.5 μ g), and Lane 3, Ni-NTA purified rTTR (0.5 μ g) were electrophoresed on a 10-15% PhastGel and stained with Coomassie blue using a PhastSystem electrophoresis and staining device (GE Healthcare). Positions corresponding to the bands observed for molecular weight markers (Bio-Rad) in kDa are indicated.

Fig. 5.

Mass characterization of rTTR Cys10 mixed disulfides. Purified rTTR was *S*-sulfonated or *S*-cysteinylation by incubating for 15 h in 100 mM potassium chloride, 1 mM EDTA, 10 mM potassium phosphate, pH 7.0 containing either 40 mM sodium tetrathionate, or 400 μ M cystine. The reaction products were then analyzed by nanospray MS.

Fig. 6.

Characterization of secondary structure of rTTR products. Far-UV circular dichroism spectra were recorded at 25 °C for purified unmodified rTTR (circles), rTTR-SO₃ (diamonds), and rTTR-Cys (squares) (6-8 μ M in 10 mM sodium phosphate, 1 mM EDTA, pH 7.4) on an Aviv 215 spectropolarimeter. Data were smoothed using the Aviv noise reduction routine.

Table 1

Purification of rTTR from *E. coli* M15[pREP4]^a

Purification Step	Total protein (mg) ^b	Approx. purity of product (%) ^c	Estimated product (mg)	Estimated product (mol) ^d	Recovery (%)
Cell lysate	210	24	50	3.3×10^{-6}	100
MK(6H)GH-rTTR	42	100	42	2.8×10^{-6}	85
rTTR	42	100	42	3.1×10^{-6}	94

^aThis preparation was from 500 ml of culture, 3.76 g wet weight cells.

^bTotal protein was determined by BCA assay for lysate and A280 ($E^{1\%}_{1\text{cm}}=14.1$) for MK(6H)GH-rTTR and rTTR.

^cApprox. purity was determined by densitometric scanning of SDS-PAGE.

^dCalculated from monomer M_r : MK(6H)GH-rTTR, 15036.82; rTTR, 13760.41.