Site-specific insertion of selenium into the redox-active disulfide of the flavoprotein augmenter of liver regeneration

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A B S T R A C T

Augmenter of liver regeneration (sfALR) is a small disulfide-bridged homodimeric flavoprotein with sulfhydryl oxidase activity. Here, we investigate the catalytic and spectroscopic consequences of selectively replacing C145 by a selenocysteine to complement earlier studies in which random substitution of ~90% of the 6 cysteine residues per sfALR monomer was achieved growing Escherichia coli on selenite. A selenocysteine insertion sequence (SECIS) element was installed within the gene for human sfALR. SecALR2 showed a spectrum comparable to that of wild-type sfALR. The catalytic efficiency of SecALR2 towards dithiothreitol was 6.8-fold lower than a corresponding construct in which position 145 was returned to a cysteine residue while retaining the additional mutations introduced with the SECIS element. This all-cysteine control enzyme formed a mixed disulfide between C142 and β-mercaptoethanol releasing C145 to form a thiolate-flavin charge transfer absorbance band at ~530 nm. In contrast, SecALR2 showed a prominent long-wavelength absorbance at 585 nm consistent with the expectation that a selenolate would be a better charge-transfer donor to the isoalloxazine ring. These data show the robustness of the ALR protein fold towards the multiple mutations required to insert the SECIS element and provide the first example of a selenolate to flavin charge-transfer complex.

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Introduction

Augmenter of liver regeneration (ALR)[1] is a FAD-linked flavoprotein that functions as a sulfhydryl oxidase and a cytokine [1–6]. It exists in two spliced forms: the long-form ALR (lfALR) is involved in the disulfide bond formation pathway in the mitochondrial intermembrane space [6–8], while the short-form (sfALR) is implicated in liver regeneration [4,5]. Both forms share a core flavin-binding domain, which contains a redox-active CXXC motif proximal to the isoalloxazine ring [2,3,9,10]. The first of these cysteine residues (C142) faces the solvent and participates in disulfide exchange reactions with thiol substrates (Fig. 1). Its redox partner, C145, communicates directly with the flavin; its sulfur atom is ~3.2 Å from the C4a position of the isoalloxazine ring [2,9,10]. A schematic representation of the flow of reducing equivalents in sfALR from thiol substrate to terminal oxidant is shown in Fig. 1 [3,6,10,11].

We recently demonstrated that it is possible to randomly substitute sulfur for selenium in sfALR [12]. In that study, we characterized this selenium-containing sfALR and demonstrated that the structure of sfALR was essentially unaltered by the substitution of 90% of the sulfur atoms by selenium. Intriguingly, a new long-wavelength intermediate species was observed in the UV–VIS spectrum when DTT was added to the selenium-containing protein. Such a spectral feature might represent a charge-transfer interaction between a selenolate (E-Se$^-$) at position 145 and the flavin ring [12]. However the extensive substitution of sulfur for selenium in all the cysteines and methionines of sfALR made this assignment tentative. Here we report investigations of this long-wavelength feature by site-specific incorporation of selenocysteine (Sec, U) at position 145 of sfALR (Fig. 1). This C145U mutation allowed us to differentiate the spectral features that arise from the individual contribution of a CXXU motif from those made by the predominant population of UXXU generated when sulfur is indiscriminately substituted for selenium in sfALR [12]. In addition, the C145U mutation allowed the catalytic consequences of substitution of a selenium at the charge-transfer thiol to be investigated without confounding additional insertion of selenocysteine and selenomethionine associated with overall substitution of sulfur to selenium.
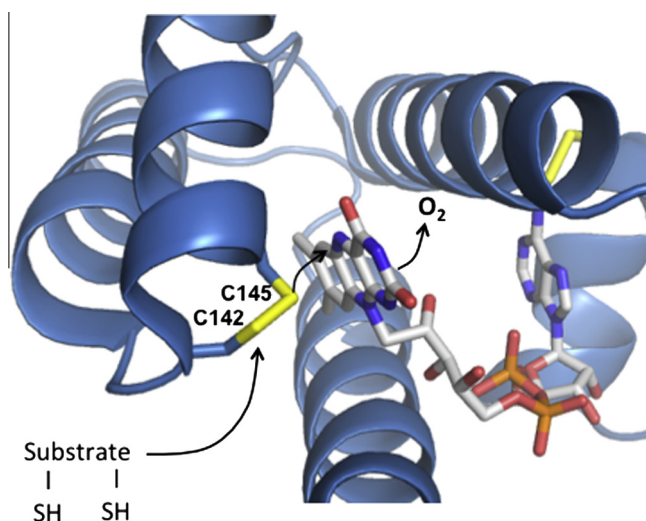
Fig. 1. Flow of reducing equivalents in the active site of mammalian ALR. The isoalloxazine ring is bound at the mouth of a bundle of four helices. C142 forms mixed-disulfide intermediates with thiol substrates. Upon scission of the proximal disulfide, C145 interacts with the oxidized flavin via a charge-transfer complex. The structure shown is that of the human sfALR (PDB 3MBG).

In order to insert Sec at position 145, we employed the native genetic incorporation machinery of *Escherichia coli*, which relies on the authentication of the UGA codon as Sec-encoding rather than as a termination codon (Fig. 2A) [13,14]. Here, a special hairpin RNA loop, termed the selenocysteine insertion sequence (SECIS), recruits the Sec-specific elongation factor (SelB) and tRNA^{Sec} to mediate the Sec insertion [15]. To communicate with SelB, the SECIS stem-loop must be placed in the immediate vicinity of the Sec codon (UGA) and carry specific nucleotides in its upper stem [15–17]. To accommodate the requirements associated with the SECIS element, genetic incorporation of Sec into sfALR in *E. coli* required introducing several mutations to the primary amino acid sequence of sfALR.

Here we report the successful expression and purification of a C145U mutant of sfALR. While the protein was generated at modest levels, it proved stable and enzymatically active with the model substrate dithiothreitol (DTT). Spectrophotometric experiments provided clear evidence that the long wavelength band, previously observed by random selenium incorporation, was a charge-transfer interaction between U145 and the oxidized flavin prosthetic group of ALR.

Materials and methods

Reagents

Chemicals and reagents were supplied by Sigma–Aldrich, Acros Organics, Fisher Scientific, GE Healthcare Bio-Sciences and GoldBio as before [11,12]. Enzymes used for molecular biology were acquired from New England Biolabs. The pSUABC plasmid was generously provided by Professor Arnér from the Karolinska Institutet [18].

Expression plasmids

A human sfALR gene (GenBank AAH28348.2) was codon optimized for expression in *E. coli* and the gene synthesized by DNA2.0. The gene (SecALR1) was provided in the expression vector pJexpress414 fused to a C-terminal His$_6$ affinity tag. The following

primers (obtained from IDT or Sigma) were used for subcloning the SecALR1 gene into the pTrcHisA and pET-28a vectors: (NheI site) 5′- AAA TTT GCT AGC ATG CGC ACC CAA CAA -3′ and (HindIII site) 5′- ACC GAA AAG CTT AAT CGC AGG AAC CG -3′. SecALR2 was generated using site-directed mutagenesis with SecALR1 as the template. The following mutagenesis primers were used for converting SecALR1 to SecALR2: 5′- GAC CTG GTT GCA CGC ACC GGA ACC AC -3′ and 5′- GTG GTT CCG GTC CGT GCA ACC AGG TC -3′. To generate the SecALR2 U145C the following mutagenesis primers were used: 5′- CCG TGT GAG GAG TGC GCT GAA GAC CTG G -3′ and 5′- CCA GGT CTT CAG CGC ACT CCT CAC ACG G -3′. The rationale for the design of both SecALR constructs is presented in the Results section. Additionally, the programs SECISDesign [19] and RNAfold [20] aided in SECIS element construction. The amino acid and nucleotide sequences of all constructs are provided in Supplementary Figs. S1 and S2.

Expression and purification of SecALR1 and SecALR2

For protein expression, the appropriate plasmid was co-transformed into *E. coli* BL21(DE3), with the pSUABC plasmid expressing *E. coli* SelA, SelB, and SelC under the control of their endogenous promoters in order to increase Sec incorporation [18]. Cells were grown in 1.2 L aliquots of Terrific Broth that were inoculated with 10 mL overnight cultures, with 100 μg/mL ampicillin (to maintain the pJexpress414 vector) and 34 μg/mL chloramphenicol (to maintain the pSUABC vector). Cells were grown at 37 °C to an OD$_{600}$ of ~2.2 and then the temperature was reduced to 18 °C approximately 30 min prior to induction. Protein expression was induced with 1 mM IPTG added with 5 μM Na$_2$SeO$_3$ and 100 μg/mL L-cysteine. The cells were grown for 24 h, harvested at 5000 g for 10 min and re-suspended in 50 mM potassium phosphate (pH 7.5) supplemented with 500 mM NaCl (binding buffer) before freezing. Purification was as previously reported [12] with the exception that approximately 100 μL of Ni-IDA (Invitrogen) resin was used per 3 L of culture medium to minimize non-specific binding. The resin was washed with 10 mL of binding buffer, followed by five 1 mL volumes of binding buffer containing 20 mM imidazole, four 1 mL volumes containing 200 mM imidazole, two 1 mL volumes containing 500 mM imidazole and two 1 mL volumes containing 1 M imidazole. Yellow fractions were combined and dialyzed against 50 mM potassium phosphate, pH 7.5, containing 1 mM EDTA. Following dialysis, the sample was analyzed for purity by reducing and non-reducing 16% tris–tricine SDS–PAGE. SecALR2 showed a final purity, as determined by SDS–PAGE analysis, of about 90%. The minor contaminant was chloramphenicol acetyltransferase, a 25 kDa protein that binds to IMAC resins. Chloramphenicol acetyltransferase does not bind cofactors and has no competing activity. Expression and purification of the all-cysteine control, SecALR2 U145C, in the pJexpress414 vector was similar to that of the wild-type protein, as previously reported [9]. The all-cysteine control was obtained in >95% purity. All enzymes, like the wild-type protein, were stable at 4 °C in 50 mM potassium phosphate (pH 7.5) containing 1 mM EDTA.

Spectral analysis

UV–VIS spectra were recorded using an HP8453 diode-array spectrophotometer (Hewlett–Packard, Palo Alto, CA). Where necessary, turbidity correction software supplied with the diode-array spectrophotometer was used to correct for slight light-scattering. Spectrophotometric experiments with β-mercaptoethanol (βME) utilized ~30 μM ALR with 20 mM thiol in either 50 mM phosphate buffer, pH 7.5, or 20 mM Tris, pH 9.0. Both buffers contained 1 mM EDTA.