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# Chemical disulfide mapping identifies an inhibitor cystine knot in the agouti signaling protein

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**Abstract** **The agouti signaling protein (ASIP) and its homolog, the agouti-related protein (AgRP), act as inverse agonists that control, respectively, pigmentation and metabolic function in mammals. NMR investigations find that the C-terminal domains of these proteins adopt a fold consistent with an inhibitor cystine knot (ICK), previously identified in invertebrate toxins. Although these structural studies suggest that ASIP and AgRP define a new mammalian protein fold class, the results with ASIP are inconclusive. Here, we apply direct chemical mapping to determine the complete set of disulfide linkages in ASIP. The results demonstrate unequivocally that ASIP adopts the ICK fold and thereby supports a recent evolution structure function analysis, which proposes that ASIP and AgRP arose from a common antagonist ligand.**

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*Keywords:* Agouti-related protein; Melanocortin receptor; Pigmentation; *tris*(2-carboxyethyl)phosphine

## 1. Introduction

Binding of the agouti signaling protein (ASIP) to the melanocortin-1 receptor (MC1R) in the skin results in production of the yellow/red pigment pheomelanin [1,2]. Mice with the agouti lethal yellow mutation ($A^y$/a) express ASIP ubiquitously, and the interaction of ASIP with the MC4 receptor in the hypothalamus causes obesity and metabolic characteristics associated with type II diabetes, including insulin and leptin resistance [3].

There are several unique and important aspects of ASIP's structure and biochemical behavior. First, melanocortin receptors (MCRs) are members of the G-protein coupled receptor (GPCR) family and ASIP, along with its homolog the agouti-related protein (AgRP), which is normally expressed in the brain, are the only known endogenous GPCR antagonists (or inverse agonists); both ASIP and AgRP act to block the action of the agonist α-MSH (reviewed in Ref. [4]). Second, whereas many GPCR ligands are small, easily diffusible peptides or low molecular weight species, ASIP is a 110 residue (after removal of the signal peptide) glycoprotein with a folded, cysteine-rich C-terminal domain [5]. Third, because of the hyperphagia and metabolic characteristics associated with $A^y$/a mice, these animals, and those with similar mutations, are widely used as obesity models [1].

ASIP's forty residue, Cys-rich, C-terminal domain alone contains the determinants for high affinity binding to MC1/4R [6,7]. Structural studies of this domain identify yet another potentially unique ASIP feature – the 10 cysteines are thought to form a network of five disulfide bonds arranged in an inhibitor cystine knot (ICK), or knottin, fold motif previously found in invertebrate toxins [6,8,9]. Characterization of this fold in the homolog AgRP is now well established by both chemical mapping [10] and NMR structural studies [11].

The specific fold of ASIP, however, is less clear. NMR studies on ASIP(80–132) by our lab identified a low energy backbone conformation quite similar to that of AgRP and consistent with the ICK motif [6]. But studies on structurally related toxins demonstrate that backbone fold alone is insufficient to define ICK disulfide bond pairings (e.g., Ref. [12]). NMR contacts in ASIP unambiguously identified disulfide bonds Cys93–Cys108, Cys116–Cys123 and Cys111–Cys132 by NOEs between specific side-chain β protons [6]. However, Cys side-chains 100, 107, 114 and 125 are all in near proximity thus confounding direct disulfide assignment by visual inspection or by side chain NOEs. The situation is further complicated by *cis*/*trans* isomerization of the Ala104–Pro105 imide backbone bond, which gives two distinct orientations of the ASIP(80–132) N-terminal loop, and structural heterogeneity for residues 100–106. Oftentimes energy calculations provide a reliable means for identifying the correct disulfide network. Specifically, DYANA calculations performed on all possible disulfide arrangements give the lowest target function only for the backbone conformation with the correct linkages [13]. This approach was attempted with ASIP and, unfortunately, failed to clearly identify a unique low energy fold, applicable to both *cis* and *trans* conformations [6].

A recent study using Evolution Structure Function analysis suggests that AgRP and ASIP arose from a common antagonist ligand that originally served to control both pigmentation and energy balance as a means for adapting to starvation [14]. In light of this proposal, and the fundamental structural issues discussed above, elucidating the exact ASIP fold is vital for establishing whether indeed AgRP and ASIP comprise a unique motif newly identified in mammals. To address these issues, we apply direct chemical mapping to determine the exact disulfide arrangement in ASIP. The method employs

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*Abbreviations:* ASIP, agouti signaling protein; MCR, melanocortin receptor; TCEP, *tris*(2-carboxyethyl)phosphine; NEM, *N*-ethylmaleimide

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alkylation of nascent sulfhydryl groups in partially reduced forms of ASIP, followed by trypsin digestion and tandem MS sequencing. These data, in conjunction with the recently determined NMR structure, provide an unequivocal classification of the ASIP fold.

## 2. Materials and methods

### 2.1. Solid phase protein synthesis

In previous work, C-terminal ASIP was prepared as two separate strands, which were then linked by native chemical ligation [6]. For these current studies, ASIP(80-132, Q115Y, S124Y), referred to as ASIP-YY, was prepared as a single strand using an Applied Biosystems 433A synthesizer and standard Fmoc chemistry. Fmoc-Cys(Trt)-OPfp was used to avoid enantiomerization of the α-carbon. Purification and oxidative folding followed the exact same procedures described previously [6].

### 2.2. Partial reduction and alkylation of nascent cysteine residues

Partial reduction was initiated by introducing ASIP-YY (1.0 mM) into a pH 3 solution containing 40 mM *tris*(2-carboxyethyl)phosphine (TCEP) and 100 mM sodium citrate [15]. After 20 min the solution was diluted with distilled water and injected into an HPLC fitted with a C18 column (Alltech's Alltima). Each partially reduced fraction was collected and lyophilized overnight for future usage. In partially reduced fractions, nascent cysteines were alkylated with *N*-ethylmaleimide (NEM) using a pH 3 solution of 100 mM NEM and 100 mM sodium citrate. The reaction time was typically 20 min. Products were repurified by HPLC.

### 2.3. Trypsin digestion and analysis by mass spectrometry

Samples were first treated dithiothreitol (DTT) to eliminate any remaining disulfide bonds. Specifically, peptides were treated for 2 h with 5 mM DTT, 6.0 M guanidine hydrochloride (GuHCl) in 100 mM 2-amino-2-hydroxymethyl-1,3-propanediol (Tris) solution at pH 8. Next, a 50 mM $NH_4HCO_3$ solution was added to dilute the GuHCl concentration to less than 1 M. Trypsin digestion used reagents and procedures from Promega. Trypsin was used in a protease to protein ratio of 1:20 and incubated at 37 °C for 4 h. The mixture was diluted with distilled water and purified by HPLC using a C18 column. Each fraction was collected for mass and sequence analysis. Mass spectrometry was performed using either a Micromass ZMD 4000 or a Thermo Finnigan LTQ. $MS^n$ experiments were performed with the LTQ.

## 3. Results and discussion

### 3.1. Disulfide mapping

Chemical disulfide mapping was performed on the stable double mutant ASIP(80–132, Q115Y, S124Y), referred to as ASIP-YY [6]. Under oxidizing conditions, ASIP-YY folds to a uniform product, as determined by both HPLC and MS, and is fully active at the MC1/3/4 receptors, with the same potency as the wild-type protein [6]. TCEP was used to gently and selectively reduce disulfide bonds [10,15]. Reduction at acidic pH avoids disulfide rearrangement. Partially reduced species were then separated by HPLC. Next, treatment of each separated species with the alkylating agent NEM labeled the free sulfhydryl groups, and this was followed by complete reduction using DTT. The selectively labeled peptides were subjected to trypsin proteolysis, which cleaves after basic residues (primarily Arg in ASIP), and the resulting fragments were sequenced by tandem MS to locate each NEM labeled thiol group.

Fig. 1 shows an HPLC trace following treatment with TCEP. Peaks b–h correspond to distinct partially reduced species. Starting with peak b, mass analysis of the NEM reacted product shows that this species contained two free sulfhydryl groups. Trypsin treatment, followed by MS analysis of the resulting fragments showed that the peptides ASIP-YY(98–117) and ASIP-YY(127–132) each contained a single NEM modification. The latter of these two peptides contains only a single Cys, thus locating one of the disulfide bond partners to Cys132. By contrast, ASIP-YY(98–117) contains six Cys, thus requiring direct sequencing to locate the modified residue.

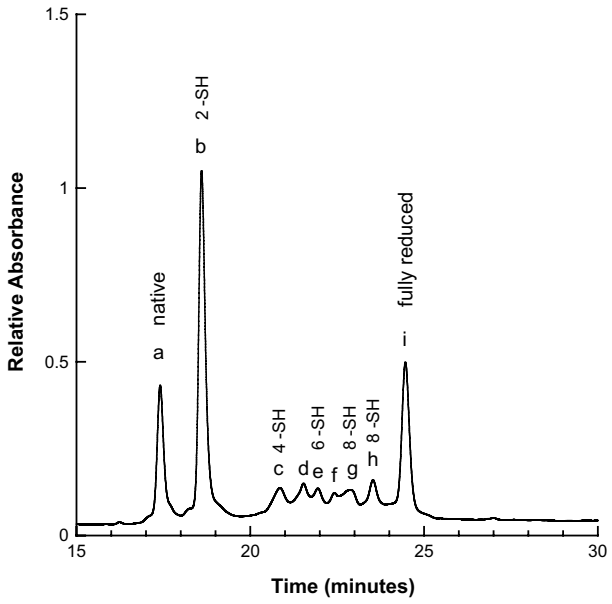


Fig. 1. HPLC trace of partially reduced ASIP-YY. The number of free –SH groups in each fraction is labeled and was determined by reaction with NEM, followed by mass analysis.

MS/MS of singly NEM modified ASIP-YY(98–117) from peak b, shown in Fig. 2A, reveals a progression of signals consistent with a loss of single amino acids cleaved at the peptide bond. Typical of trypsin digests, y ions are enhanced due to the positively charged residue at their C-termini. Sequencing from the N-terminus through residue 109 showed that the modified Cys resided in the segments 110–117. Further fragmentation was not observed so the ASIP-YY(110–117) $y_8$-ion (1027.18 amu) was maintained in the MS trap for further MS/MS/MS analysis, as shown in Fig. 2B. Following loss of N-terminal Pro110, sequential fragmentation of the $y_7$-ion gave a change in mass from 930.33 amu to 702.30 amu, corresponding to an NEM modified cysteine, thus locating the other disulfide bond partner to Cys111. Loss of additional amino acids was consistent with y-ion fragmentation of the unmodified peptide ASCYCR. Taken together, these data demonstrate that ASIP-YY contains a disulfide bond between Cys111 and Cys132, as found in the NMR structure.

HPLC peaks c, e, g and h, with four, six, eight and eight NEM modifications, respectively, were subjected to the same digestion and MS analysis, thus locating all pairwise NEM labeled Cys residues. Peaks d and f were heterogeneous and therefore not used in our analysis. The results are summarized in Fig. 3. Here, Cys residues colored with a blue background identify NEM labeling. The vertical red lines indicate observed trypsin cleavage sites. Peak c revealed four NEM modifications; two confirmed labeling at Cys111 and Cys132

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