Physiol Genomics 20: 12–14, 2004.
First published October 26, 2004; doi:10.1152/physiolgenomics.00204.2004.

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Altered specificity of Hint-W123Q supports a role for Hint inhibition by ASW in avian sex determination

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Submitted 7 September 2004; accepted in final form 25 October 2004

Parks, Kristen P., Heather Seidle, Nathan Wright, Jeffrey B. Sperry, Pawel Bieganowski, Konrad Howitz, Dennis L. Wright, and Charles Brenner. Altered specificity of Hint-W123Q supports a role for Hint inhibition by ASW in avian sex determination. *Physiol Genomics* 20: 12–14, 2004. First published October 26, 2004; doi: 10.1152/physiolgenomics.00204.2004.—Hint is a universally conserved, dimeric AMP-lysine hydrolase encoded on the avian Z chromosome. Tandemly repeated on the female-specific W chromosome, Asw encodes a potentially sex-determining, dominant-negative Hint dimerization partner whose substrate-interacting residues were specifically altered in evolution. To test the hypothesis that Gln127 of Asw is responsible for depression and/or alteration of Hint enzyme activity, a corresponding mutant was created in the chicken Hint homodimer, and a novel substrate was developed that links reversal of AMP-lysine modification to aminomethylcoumarin release. Strikingly, the Hint-W123Q substitution reduced k_{cat}/K_m for AMP-lysine hydrolysis 17-fold, while it increased specificity for AMP-*para*-nitroaniline hydrolysis by 160-fold. The resulting 2,700-fold switch in enzyme specificity suggests that Gln127 could be the dominant component of Asw dominant negativity in avian feminization.

AMP-lysine hydrolase; W chromosome; dominant negative; site-directed mutagenesis

HINT IS A HOMODIMER of ∼14 kDa subunits that functions as an AMP-lysine hydrolase and positive regulator of Kin28 in yeast, whose most conserved amino acids form the dimer interface and the substrate binding site (2, 3, 8). The Hint active site consists of mostly nonpolar residues that contribute to adenosine binding (3), the histidine that forms a phosphoramide with the substrate α-phosphate (11), Ser107, which interacts with the leaving group amine (8), and Trp123, which interacts with the alkyl portion of the lysine leaving group across the dimer interface (8).

Although it is known that male birds are homogametic with a ZZ karyotype and females are heterogametic with a ZW karyotype, the molecular basis for sexual differentiation is unknown, although the existence of a ZZW female warbler strongly suggests that genetic information on the W chromosome is responsible for feminization (1). In birds other than ostriches and emus, the female-specific W chromosome carries ∼40 tandem repeats of an unusual Hint-related gene, *ASW*, whereas the Z chromosome carries a typical *HINT* gene (7, 12). In a striking departure from all previously isolated Hint homologous sequences, which conserve the AMP-lysine binding site more than other residues, the female-specific Asw protein has strong similarity to Hint except that 15 of 16 substrate-interacting residues are sexually dimorphic, i.e., altered in the W-encoded Asw with respect to the Z-encoded Hint (13). Thus, because the predicted dimerization interface (helix α2 and beta strand β4) is virtually unaltered in Asw (13), and a single His-to-Ala substitution can reduce the catalytic activity of Hint by over 100,000-fold (2), evolutionary pressures may have ablated the AMP-lysine binding site in Asw but allowed Asw to function as a Hint heterodimerization partner. In this regard, it is important to note that of the 16 substrate-interacting residues in the Hint dimer, only one interacts with the AMP-lysine substrate across the dimer interface (8). That residue, Trp123, in the COOH-terminal Trp-Pro-Pro-Gly motif of Hint, is conspicuously substituted by Gln in the repeated, female-specific Asw sequence (13).

As a candidate female sex-determining gene, we reasoned that dominance and negativity might be functionally separable components of Asw's activities. Negativity would seem to be a function of loss of AMP-binding residues in the Asw sequence, but, because the Hint dimer is not cooperative with respect to substrate hydrolysis (2), an inert dimerization partner would fail to depress Hint enzymatic activity by more than 50%. In fact, because there is no *HINT* gene on the W chromosome, *HINT* gene dosage is already reduced 50% by *HINT* gene absence. We therefore considered an explanation necessary for why a potential Hint dimerization partner lacking an active site is repeated 40 times on the W chromosome. By constructing a model of the putative Hint-Asw heterodimer based on crystal structures of rabbit Hint bound to products (3) and substrate analogs (8), we noted that Hint Trp123 is expected to be located on the Asw side of the dimer interface (13). More importantly, the model predicted that the residue in Asw that corresponds to Hint Trp123, namely Gln127, is physically located in the Hint half of the putative heterodimer. Thus we proposed that Gln127 in place of Trp123 is responsible for dominant depression and/or dominant alteration of activity of the Hint active site (13). In this study, with site-directed mutagenesis of chicken Hint and synthesis of a novel fluorescent Hint substrate, we establish that the W123Q allele of Hint creates a 2,700-fold alteration of substrate specificity,

Article published online before print. See web site for date of publication (http://physiolgenomics.physiology.org).

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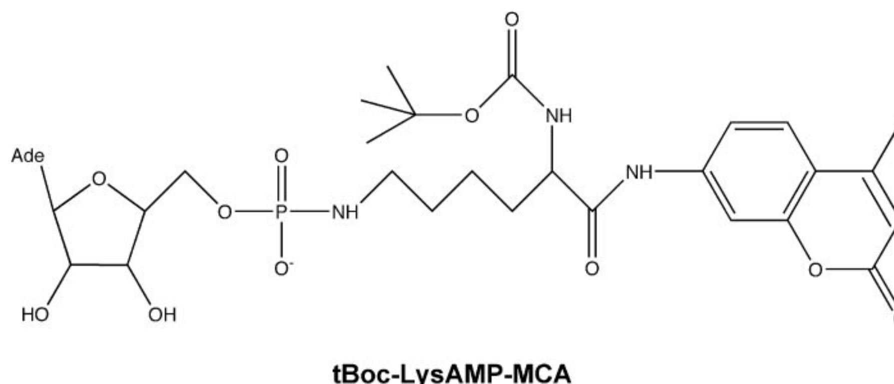

Fig. 1. Structure and synthesis of tBoc-LysAMP-MCA. Because the ϵ-adenylyl modification of Lys blocks trypsin cleavage, Hint enzymatic activity is limiting for production of *tert*-butoxycarbonyl-L-lysine methylcoumarinamide (tBoc-Lys-MCA), a trypsin substrate (see Supplemental Material, at the *Physiological Genomics* web site). tBoc-LysAMP-MCA was made as a modification of the adenosine 5′-phosphoramidate synthesis method of Fu et al. (4). Under an argon atmosphere at room temperature, 0.25 mmol of tBoc-Lys-MCA (Bachem) and 0.12 mmol ADP (Sigma) were dissolved in 2 ml pyridine plus 0.6 ml trimethylsilyl chloride, added dropwise, and the resulting mixture was stirred for 2 days. The mixture was evaporated, and 1 ml of 2 M aqueous ammonia was added to hydrolyze the residue. Product was extracted with four 5-ml volumes of diethyl ether, evaporated to dryness, and dissolved in 1.5 ml isopropanol:2 M aqueous ammonia:methanol (7:1:2). tBoc-LysAMP-MCA (yield = 20.6%) was purified twice by silica gel column chromatography using a 10 × 1 cm column. Peak material was analyzed by matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) and nuclear magnetic resonance (NMR). MALDI was performed on an Applied Biosystems Voyager system 6235 in negative ion mode using 3-hydroxypicolinic acid (observed mass = 733.79; calculated mass = 734.26). ^{31}P, ^{13}C, and ^{1}H-NMR data were collected using a 15-mm probe with the compound in DMSO. ^{1}H-NMR (500 mHz, DMSO-d_6, 50°C) δ = 10.93 (s, 1H), 10.85 (s, 1H), 8.47 (s, 1H), 8.12 (s, 1H), 7.81 (d, J = 2.0 Hz, 1H), 7.71 (d, J = 8.5 Hz, 1H), 7.53 (d, J = 8.8 Hz, 1H), 7.35–7.44 (m, 2H), 7.27 (d, J = 7.6 Hz, 1H), 7.10–7.16 (m, 1H), 6.25 (d, J = 1.2 Hz, 1H), 5.89 (d, J = 5.4 Hz, 1H), 5.57–5.62 (m, 1H), 4.55–4.59 (m, 1H), 4.18–4.21 (m, 1H), 4.07–4.21 (m, 1H), 4.02–4.05 (m, 1H), 3.83–3.88 (m, 1H), 3.74–3.80 (m, 1H), 2.72–2.77 (m, 1H), 2.66–2.70 (m, 1H), 2.36 (d, J = 4.2 Hz, 3H), 1.64–1.70 (m, 1H), 1.57–1.61 (m, 1H), 1.33 (d, J = 13.9 Hz, 9H), 1.23–1.25 (m, 1H), 1.00 (d, J = 6.1 Hz, 3H); ^{13}C-NMR (hmqc, DMSO-d_6, 50°C, partial) δ = 153.3, 126.5, 116.0, 112.7, 106.2, 87.5, 84.4, 74.5, 71.3, 64.3, 41.5, 40.3, 31.6, 28.8, 28.6, 28.1, 26.0, 18.6; ^{31}P-NMR (300 mHz, DMSO-d_6) δ = 2.66.

supporting a mechanistic role for Asw's COOH-terminal Gln in feminization of developing birds.

EXPERIMENTS AND RESULTS

To study the AMP-lysine hydrolase activity of wild-type chicken Hint and the Hint-W123Q mutant, we modified a fluorigenic trypsin substrate [*tert*-butoxycarbonyl-L-lysine methylcoumarinamide (tBoc-Lys-MCA)] by adenylylation of the ϵ-amino group of lysine (tBoc-LysAMP-MCA, Fig. 1). Because this modification renders the lysinamide moiety resistant to trypsin, Hint incubations to liberate tBoc-Lys-MCA are coupled to tryptic digestion to quantitate Hint activity with aminomethylcoumarin (AMC) release from tBoc-Lys (see Supplemental Materials, are available online at the *Physiological Genomics* web site).[1] As shown in Fig. 2 and Table 1, wild-type chicken Hint hydrolyzed tBoc-LysAMP-MCA with a k_{cat} of 24 s^{-1} and a K_m of 6.1 μM (specificity constant = 3,950,000 $M^{-1}s^{-1}$). Consistent with a dominant-negative role for the COOH-terminal Gln of Asw, the Hint-W123Q substitution depressed k_{cat} ninefold (to 2.6 s^{-1}) and increased K_m twofold (to 11 μM) for an overall 17-fold decline in k_{cat}/K_m (229,000 $M^{-1}s^{-1}$). Because an Asw-Hint heterodimer would have only a single Hint active site per dimer, the predicted depression in AMP-lysine hydrolytic activity is >30-fold.

[1]The Supplemental Material for this article (Supplemental Methods and Figs. S1–S3) is available online at **http://physiolgenomics.physiology.org/cgi/content/full/00204.2004/DC1**.

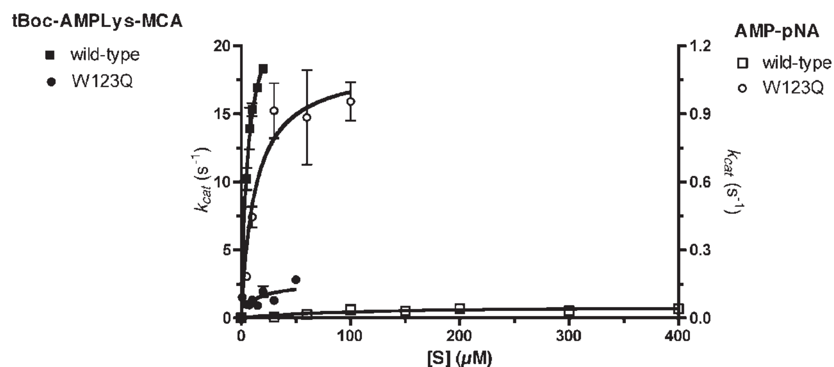

Fig. 2. The 2,700-fold alteration of substrate specificity by W123Q alteration. The chicken Hint cDNA was amplified from EST clone pat.pk0069.a10 from the University of Delaware using primers 7038 (5′ GATCGTCCATATGGCTGACGAGATCCGCAAGG) and 7039 (5′ CACTCTCGAGTTAGCCAGGAGGCCAGCCCAACTG) and cloned into pSGA02 as an *Nde*I-*Xho*I fragment. The W123Q substitution was introduced (9) with mutagenic primer 7046 (5′ GGTCGTCAGTTGGGCCAGCCTCCTGGCTAA). Enzymes were purified as described (2, 8). tBoc-LysAMP-MCA assays, at 5–50 μM, described and validated in detail in Supplementary Materials, were performed with 1.8 fmol of wild-type or 19 fmol Hint-W123Q at pH 5.5 with 1 mM EDTA. Reactions (10–20 min) were stopped by adjusting the pH to 9.5, addition of trypsin to a final concentration of 60 μg/μl to liberate aminomethylcoumarin (AMC), then quantitated by fluorescence. AMP-*para*-nitroaniline (AMP-pNA) assays with 3.6 pmol wild-type or 0.36 pmol Hint-W123Q were as described (8). Solid symbols are on the *left* y-axis. Open symbols are on the *right* y-axis.

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Table 1. *Kinetic constants for wild-type and W123Q chicken Hint*

	tBoc-LysAMP-MCA			AMP-pNA		
	k_{cat}, s^{-1}	K_m, μM	k_{cat}/K_m, $M^{-1}s^{-1}$	k_{cat}, s^{-1}	K_m, μM	k_{cat}/K_m, $M^{-1}s^{-1}$
Wild type	24.1±1.56	6.14±1.11	3,950,000	0.051±0.011	93.0±57.6	549
W123Q	2.57±0.473	11.2±5.11	229,000	1.12±0.168	12.7±7.27	88,200

tBOC-LysAMP-MCA, *tert*-butoxycarbonyl-L-lysine methylcourinamide, with adenylylation of the ε-amino group of lysine; AMP-pNA, AMP-*para*-nitroaniline.

Conversion of Hint dimers to Asw-Hint heterodimers could either depress AMP-lysine hydrolytic activity, promote another activity, or both. To test whether substitution of Trp123, which makes a hydrophobic interaction with the Lys leaving group of AMP-lysine in the substrate across the dimer interface (8), would promote hydrolysis of a bulkier adenylylated phosphoramide substrate, we tested wild-type and W123Q forms of chicken Hint on AMP-*para*-nitroaniline (AMP-pNA) (8). This molecule, which is followed spectroscopically, is a poor substrate for wild-type rabbit Hint (8). In the chicken system, the wild-type k_{cat} was depressed 470-fold (to 0.051 s^{-1}) and K_m was elevated 15-fold (to 93 μM), for an overall specificity constant of 550 $M^{-1}s^{-1}$ with AMP-pNA. Remarkably, Hint-W123Q is 160-fold superior AMP-pNA hydrolase compared with wild-type Hint (k_{cat} = 1.1 s^{-1}; K_m = 13 μM; k_{cat}/K_m = 88,000 $M^{-1}s^{-1}$). The 17-fold depressed AMP-lysine hydrolase activity coupled with 160-fold superior AMP-pNA hydrolase activity represents a 2,700-fold alteration of specificity, which is remarkable for a single amino acid substitution.

DISCUSSION

Although sex-specific body plans are fundamentally similar in all vertebrate phyla, birds and mammals make use of unique chromosomal sex determination systems while crocodiles, many turtles and some lizards lack genetically encoded switches and make use of environmental sex determination (5). Thus it has been argued that genetic switches have evolved independently in birds and mammals that take the place of temperature-dependent switches in organisms without sex chromosomes. Because Asw is repeated 40 times on the female-specific W chromosome and is highly expressed at the urogenital ridge precisely at the time of feminization (7, 12), we have considered Asw to be a strong candidate gene for female sex determination (13). The ability of Asw residue 127 to repress and alter Hint enzymatic activity is a plausible mechanism for function of Asw in this process; either of these activities might be sufficient for Asw function. Because functions of Hint that depend on protein-protein interactions have been proposed (10), one could hypothesize that Asw functions by altering putative Hint complexes or promoting a female-specific complex. However, as a Hint active site mutant is a functional null (2), it was interesting to determine whether an Asw residue could repress Hint enzymatic activity in *trans*.

This study is also instructive in dissection of how nature constructs a dominant-negative allele. As described by Ira Herskowitz (6), a dominant-negative allele ought to have two characteristics: simple loss of function and dominant interference. In the case of Asw, loss of function can be attributed to mutation of the active-site residues (13), which is sufficient to produce an inactive allele in vitro and in vivo (2). Because overexpression of an inert molecule would be pointless, however, we searched for the source of dominant interference in Asw. Transplantation of the dimer-crossing Trp to Gln substitution of Asw into the Hint sequence both depressed and altered specificity, consistent with a dominant-negative mechanism for Asw function in avian feminization.

ACKNOWLEDGMENTS

We thank Dr. Joan Burnside of the University of Delaware for the kind gift of EST clone pat.pk0069.a10.

GRANTS

This work was supported by National Cancer Institute Grant CA-75954 to C. Brenner.

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