Key Determinants of Receptor Activation in the *agr* Autoinducing Peptides of *Staphylococcus aureus*†

Gholson J. Lyon,‡ Jesse S. Wright,§ Tom W. Muir,*,‡ and Richard P. Novick*,§

Laboratory of Synthetic Protein Chemistry, The Rockefeller University, 1230 York Avenue, New York, New York 10021, and Molecular Pathogenesis Program, Skirball Institute of Biomolecular Medicine, New York University Medical Center, New York, New York 10016

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ABSTRACT: Staphylococcal pathogenesis is regulated by a two-component quorum-sensing system, *agr*, activated upon binding of a self-coded autoinducing peptide (AIP) to the receptor−histidine kinase, AgrC. The AIPs consist of a thiolactone macrocyle and an exocyclic "tail", both of which are important for function. In this report, characterization of the unique AIPs from the four known *agr* specificity groups of *Staphylococcus aureus* has been completed, along with analysis of cross-group inhibition of AgrC activation by each of the four AIPs. The following conclusions have been drawn: (i) The native thiolactone macrocyle and tail are necessary and sufficient for full activation by the AIPs, whereas the AIP-I macrocycle alone is a partial agonist. (ii) The native N-terminus is less critical, as that of AIP-I can be modified without affecting bioactivity, although that of AIP-III cannot. (iii) The ring and tail may function differently in different AIPs. Thus the group I and IV AIPs differ at a single (endocyclic) residue, which is the determinant of AIP specificity for these two groups and is essential for function. A similarly critical residue in AIP-II, however, is exocyclic. (iv) Cross-inhibition is more tolerant of sequence and structural diversity than is activation, suggesting that the AIPs interact differently with cognate than with heterologous receptors. (v) Chimeric peptides, in which the tails and macrocycles are switched, do not activate and instead inhibit receptor activation. These data suggest a model in which activation and inhibition involves different binding orientations within the ligand binding pocket of each receptor.

Receptor-regulated histidine kinase signaling (or two-component signaling) is widespread in nature, especially in bacteria, and is mainly utilized for cell−cell and cell−environment communication. In the vast majority of cases, the ligands or environmental cues that trigger histidine kinase signaling are unknown. Noted exceptions include amino acid ligands, such as aspartate, in the bacterial chemotaxis system (*1*) and more complex peptide ligands, such as thiolactone-containing autoinducing peptides (AIPs),[1] in the *agr* system of *Staphylococcus aureus* (*2*). A great deal is known concerning ligand recognition and signaling by two-transmembrane-helix-containing chemoreceptors in bacterial chemotaxis (*1*, *3*). In contrast, relatively little is known about peptide ligand recognition by polytopic receptor−histidine kinases (RHKs) in *S. aureus* or any other species.

The ligands of the *agr* system in *S. aureus* have been characterized and therefore represent a model system in which to elucidate the mechanistic basis for ligand-mediated RHK activation. Cell density-dependent accumulation of an extracellular peptide, known as the AIP (generated by AgrB through processing of the propeptide, AgrD), triggers AgrC activation. This leads to activation of an *agr* response via the unique regulator, RNAIII (*4*). The sequence of the AIPs is highly variable, resulting in at least four specificity groups of strains within *S. aureus* (Figure 1) and many more (>25) in other staphylococci (*5*−*8*). A group is defined by the fact that all strains within the group produce the same AIP. The *agrB*, *-D*, and *-C* coding regions vary in concert to maintain the specificity of AIP processing and function (*7*). This specificity results in four different receptors for the AIPs in *S. aureus*, designated AgrC-I, -II, -III, and -IV, reflecting the group that expresses them. Remarkably, there is extensive cross-talk at the level of ligand-mediated signaling, as most AIPs activate their native receptor while competitively inhibiting activation of non-native receptors (*7*). This inhibition is a form of bacterial interference that does not result in growth inhibition but rather in the block of accessory gene functions, presumably resulting in an advantage for the strain producing the most abundant and/or most potent AIP.

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* Corresponding authors. T.W.M.: tel, 212-327-7368; fax, 212-327-7358; e-mail, muirt@rockefeller.edu. R.P.N.: tel, 212-263-6290; fax, 212-263-5711; e-mail, novick@saturn.med.nyu.edu.

‡ The Rockefeller University.

§ New York University Medical Center.

[1] Abbreviations: RHK, receptor−histidine kinase; *agr*, accessory gene regulator; AIP, *agr* autoinducing or inhibiting peptide; *blaZ*, β-lactamase; V_{init}, initial velocity of cleavage of nitrocefin by β-lactamase; CAx-y, group *x agrC* and *agrA* on the group *y agr*-null background; RP-HPLC, reversed-phase high-pressure liquid chromatography; NMR, nuclear magnetic resonance; ESMS, electrospray mass spectroscopy; HF, hydrogen fluoride; MBHA, methylbenzhydrylamine; DMSO, dimethyl sulfoxide; *m/z*, mass/charge ratio in ESMS; EC_{50}, half-maximal effective concentration; IC_{50}, half-maximal inhibitory concentration; SEM, standard error of the mean; CI, confidence interval. Standard single and triple letter amino acid codes are used throughout.

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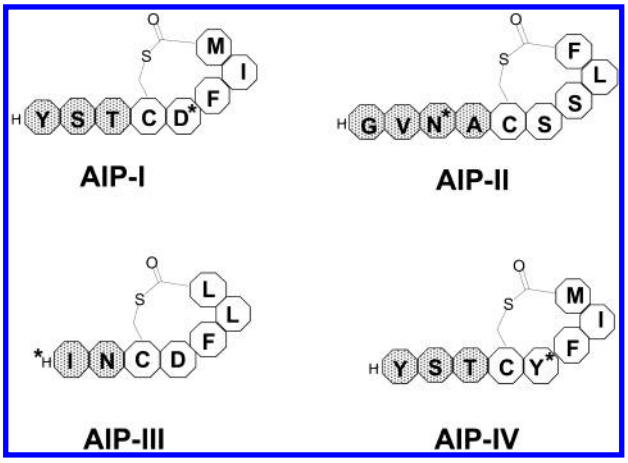

FIGURE 1: Structure of the AIPs. Standard single letter codes for amino acids are indicated. The sulfur atom of the cysteine and the carbonyl contributed from the C-terminal amino acid are shown in a thioester linkage, which closes the macrocycle. Exocyclic (tail) residues are represented in shaded gray circles, whereas endocyclic residues are represented in clear circles. Residues that are critical for receptor activation are marked with an asterisk (∗). The N-terminus of AIP-III is marked with an asterisk (∗) to reflect the fact that additional amino acids on the N-terminus abolish receptor activation.

AgrC and the AIPs are the only group-specific components of the system that are required for *agr* activation and inhibition (*9*). The N-terminal region of AgrC is called the sensor domain because it contains the agonist AIP binding site (*10*) and is the determinant of group specificity (*9*). Topological mapping and biochemical analysis suggest that the sensor domain is composed of five or six transmembrane helices, depending on whether the N-terminus of the protein is placed on the inside or outside of the cell (*10*). Agonism and antagonism of AgrC activation are reversible, and agonists and antagonists compete for an overlapping binding site on the sensor domain of AgrC (*11*). As the AIPs appear to activate or inhibit only AgrC, we will refer to activation or inhibition of each group according to the group-specific sensor domain of the activated or inhibited receptor. For example, group II activation will henceforth be referred to as AgrC-II activation, and inhibition of group I activation will be referred to as AgrC-I inhibition, thus reflecting the mechanistic basis for these processes.

Extensive structure−activity relationship (SAR) studies have been performed on the AIPs (*5*, *9*, *11*−*14*) and have shown the following: (i) The length of the AIPs varies from seven to nine amino acids, with an invariant cysteine located five amino acids from the C-terminus, which is fixed on the basis of the absolute conservation of a presumed AgrD processing motif (DE) directly following it in over 30 different AgrDs (*8*). (ii) The AIPs contain a thiolactone that is formed from the condensation of the α-carboxyl group of the peptide with the sulfhydryl group of the conserved cysteine (Figure 1). (iii) This macrocycle is required for agonist activity (residues contained within the macrocycle are referred to as endocyclic, whereas residues N-terminal to the conserved cysteine and therefore outside of the macrocycle are referred to as "tail" or exocyclic residues). (iv) The nature of the linkage forming the macrocycle, comprised of a thioester bond, is critical for certain aspects of biological activity, as the lactam analogues of AIP-I and AIP-II are potent cross-group inhibitors but activate receptors within their group only at very high concentrations (*11*, *14*). (v) The C-terminal three and two residues in AIP-I and -II, respectively, are hydrophobic, and substitution of these residues by alanine decreases activation and cross-inhibition dramatically (*12*, *14*). (vi) In the case of AIP-II, the tail is necessary for AgrC-II activation but not for AgrC-I inhibition (*12*), and substitution of asparagine for alanine at position 3 in the tail of AIP-II converts an agonist into an antagonist of AgrC-II activation (*11*). (vii) A truncated version of AIP-II, lacking the tail but retaining the thioester bond, is an *agr* inhibitor for all four *S. aureus* groups (*9*). (viii) Subsitution of the endocyclic aspartate by alanine at position 5 in AIP-I converts it from an agonist into an antagonist of AgrC-I activation (*14*).

To ascertain some of the biologically important determinants in the AIPs and to make comparisons between them, we have extended our analysis of the AIPs and analogues derived from them to the four known *S. aureus agr* groups. These studies have revealed key determinants in the AIPs that are critical for receptor binding and/or activation and have demonstrated that, across groups, these key residues are not always at the same position in the linear sequence of the AIPs. Instead, these key determinants are exocyclic in some cases and endocyclic in others and probably confer specificity for activation and cross-inhibition by binding to the receptors in different three-dimensional configurations.

EXPERIMENTAL PROCEDURES

General Methods. All amino acid derivatives and resins were purchased from Novabiochem (San Diego, CA) and Peninsula Laboratories (Belmont, CA). All other chemical reagents were purchased from Aldrich Chemical Co. Restriction enzymes were purchased from New England Biolabs (Beverly, MA) unless otherwise specified. Dimethyl-d_6 sulfoxide was purchased from Isotec Inc. (Miamisburg, OH), and nitrocefin was purchased from Becton-Dickinson (Franklin Lakes, NJ). Analytical gradient reversed-phase HPLC was performed on a Hewlett-Packard 1100 series instrument with diode array detection. Analytical HPLC was performed on a Vydac C18 column (5 μm, 4.6 × 150 mm) at a flow rate of 1 mL/min. Preparative HPLC was routinely performed on a Waters DeltaPrep 4000 system fitted with a Waters 486 tunable absorbance detector using a Vydac C18 column (15−20 μm, 50 × 250 mm) at a flow rate of 30 mL/min. All runs used linear gradients of 0.1% aqueous TFA (solvent A) vs 90% acetonitrile plus 0.1% TFA (solvent B). Mass spectrometric analysis was routinely applied to all synthetic peptides and components of reaction mixtures. ESMS was performed on a Sciex API-100 single quadrupole electrospray mass spectrometer.

Bacterial Strains and Growth Conditions. The bacterial strains used have been described in a previous study (*9*), with the exception of the group III reporter strain described below. These strains are RN9222 (CA1-I), RN9372 (CA2-II), and RN9371 (CA4-IV) and are referred to as group I, II, or IV or AgrC-I, -II or -IV, respectively. *S. aureus* cells were grown in CYGP broth (*15*) with shaking at 37 °C. Overnight cultures on GL plates (*15*) were routinely used as inocula. Cell growth was monitored with a Klett-Summerson colorimeter with a green (540 nm) filter (Klett, Long Island City, NY). Antibiotics used were erythromycin (10 μg/mL) and tetracycline (5 μg/mL).

Table 1: AIPs and Derived Analogues

AIPs	exocyclic[a]			endocyclic					mass (Da)	
									expected	observed
AIP-I		**Y**	**S**	**T**	C[b]	D	F	I	M	961.1	960.9
AIP-I D5N		**Y**	**S**	**T**	C	N	F	I	M	960.1	959.9
AIP-IV		**Y**	**S**	**T**	C	Y	F	I	M	1009.2	1008.9
AIP-IV Y5F		**Y**	**S**	**T**	C	F	F	I	M	993.2	992.9
AIP-I D5A		**Y**	**S**	**T**	C	A	F	I	M	917.1	916.9
AIP-I M8I		**Y**	**S**	**T**	C	D	F	I	I	943.1	942.9
acetyl-AIP-I	**ac**	**Y**	**S**	**T**	C	D	F	I	M	1003.2	1003.0
biotinylated AIP-I	**bi**	**Y**	**S**	**T**	C	D	F	I	M	1187.4	1186.9
fluorescein AIP-I	**fl**	**Y**	**S**	**T**	C	D	F	I	M	1319.4	1318.8
trAIP-I				**ac**	C	D	F	I	M	651.8	652.0
trAIP-IV				**ac**	C	Y	F	I	M	699.9	700.0
trAIP-I D2A				**ac**	C	A	F	I	M	607.8	607.0
AIP-II	**G**	**V**	**N**	**A**	C	S	S	L	F	879.0	878.9
trAIP-II				**ac**	C	S	S	L	F	578.7	579.0
AIP-II lactam	**G**	**V**	**N**	**A**	X[c]	S	S	L	F	861.9	862.0
AIP-II linear free acid	**G**	**V**	**N**	**A**	C	S	S	L	F	897.0	896.4
AIP-II/I	**G**	**V**	**N**	**A**	C	D	F	I	M	951.1	951.0
AIP-I/II		**Y**	**S**	**T**	C	S	S	L	F	889.0	889.0
AIP-III			**I**	**N**	C	D	F	L	L	819.0	818.9
AIP-III octapeptide		**Y**	**I**	**N**	C	D	F	L	L	982.2	981.9
AIP-III nonapeptide	**A**	**Y**	**I**	**N**	C	D	F	L	L	1053.2	1052.9

[a] Exocyclic residues are in bold in the table. Abbreviations: ac, acetylation; bi, biotinylated; fl, fluorescein conjugated. [b] All of the peptides with a conserved cysteine contain a thiolactone formed via the condensation of the α-carboxyl group of the AIPs with the sulfhydryl group, except for AIP-II linear free acid, which has a C-terminal carboxylic acid and a free thiol group. [c] X = Dapa (diaminopropionic acid).

Construction of AgrC-III−IV Chimeric Receptor. The sensor domain from one AgrC can be attached to the HK domain of a different AgrC without changing the signaling characteristics of that domain (*11*), and these receptors can be expressed with AgrA in a group I *agr*-null genetic background without the requirement of any other group-specific factors (*9*). Therefore, a chimeric receptor containing the N-terminal sensor domain of the group III *agrC* coding sequence fused to the C-terminal histidine kinase domain of the group IV *agrC*, followed by the *agrA* coding region, was assembled in the reporter construct pRN7128, previously referred to as pRN7107-2, and utilized for construction of the group I and group II chimeras (*11*). The group III sensor domain was amplified from chromosomal DNA isolated from group III strain RN8465 with *Pwo* polymerase (Roche) using the 5′ oligonucleotide JSW9, 5′-GGCGC**CTGCAG**GAAG-TACCAAAAGAATTAACTC (*Pst*I site in bold), and the 3′ oligonucleotide JSW8, 5′-TATTTCATTTC**CTTAAG**AG-TAAATTG (*Afl*II site in bold). The PCR product was digested with *Pst*I and *Afl*II and ligated into pRN7128 with the same enzymes to create the reporter pRN7131. The introduced sequence was confirmed by DNA sequencing. Plasmid pRN7131 was electroporated into the restriction-deficient *S. aureus* strain RN4220 and transduced to strain RN6911 *agr*-null (*4*) as described (*15*). This new reporter strain (RN9532 and referred to as group III or AgrC-III) uses β-lactamase production from a P3-*blaZ* fusion to monitor group III activation and lacks any endogenous AIP-III. Group III cells are induced for β-lactamase activity by culture supernatants of group III strains but not of group I, II, or IV strains, and synthetic AIP-I, -II, and -IV do not activate this cell line at concentrations up to 10 μM. Group III culture supernatants were filtered through a 10 kDa cutoff Centricon filter to remove β-lactamase, which is endogenously produced by all group III strains in our collection.

Synthetic Peptides. AIPs used in this study are listed in Table 1. All AIPs were chemically synthesized using standard solid-phase approaches or as described (*12*), except that preloaded (*tert*-butoxycarbonyl)aminoacyl-3-mercaptopropionamide−4-methylbenzhydrylamine−copoly(styrene-1% DVB) (Boc-AA-[COS]-MBHA) support was utilized instead of the previously used Boc-AA-[COS]-PEGA support. The 3-thiopropionic acid linker on MBHA resin is labile to HF-cleavage conditions, thereby releasing linear thioester peptides upon global deprotection. Following removal of HF, the crude peptide product was precipitated using cold Et_2O, washed thoroughly with Et_2O, and then dissolved in 50% CH_3CN/50% water/0.1% TFA. After lyophilization, linear thioester peptides were dissolved again in CH_3CN and water with 0.1% TFA and cyclized in solution by the addition of at least 1× volume of 0.2 M phosphate buffer at pH 7. The advantage of this solution-based approach over on-bead cyclization is that the kinetics of cyclization can be more easily monitored. In the majority of cases, >90% conversion of linear thioester to cyclic product was achieved in 3 h or less. After cyclization, the peptides were purified by RP-HPLC and characterized by analytical RP-HPLC, mass spectrometry (Table 1), amino acid analysis, and thioester base-lability studies, which collectively demonstrated that all peptides were of the correct composition and >95% pure. Two-dimensional ^1H NMR analysis yielded complete assignments for all synthetic peptides, and all chemical shift data are listed in the Supporting Information.

The concentrations of stock solutions were calculated on the basis of amino acid analysis, with the peptides dissolved in 45% CH_3CN/55% water/ 0.1% TFA. This amino acid analysis is associated with a ±10% error, as determined by multiple hydrolyses and HPLC analyses of single samples. Peptide stocks were made in 25% propylene glycol and 0.05 M phosphate, pH 5.7, at a known concentration and serially diluted into the same buffer for assays with cells. Two AIP analogues, trAIP-IV and trAIP-I D2A, were insoluble in this solvent system due to their extreme hydrophobicity. In these instances, DMSO was used initially to solubilize the peptides,

which were then serially diluted into 25% propylene glycol and 0.05 M phosphate, pH 5.7.

NMR Spectroscopy. All NMR spectra were recorded on a Bruker AMX-400 spectrometer and processed using the XWINNMR software package (Bruker). The measurements were performed at 298 K using 0.5−2.0 mM solutions of the peptides in 90% H_2O/10% D_2O, pH 4.0, or DMSO-d_6, and all chemical shifts are referenced to the solvent signal [either TMS at 0 ppm (^{1}H) or the solvent signal of DMSO-d_6 at 2.50 ppm (^{1}H)]. Assignment of resonances was achieved by 2D NMR techniques [TOCSY (*16*) and ROESY (*17*)]. Mixing times were 77 ms for TOCSY spectra and 200 ms for ROESY spectra. The chemical shifts of the amide, α, and β protons were compared between the different peptides in the same solvent system and were normalized to the shifts of the most closely related native AIP.

Agr Activation and Inhibition Assays. Assays were performed with bacterial cultures in early exponential phase (~2 × 10^8 cells/mL) and used a β-lactamase reporter gene readout. For activation assays, 80 μL of cells (9 × 10^8 cells/mL) was treated in duplicate with AIPs or AIP analogues at varying concentrations and incubated with shaking at 37 °C for 60 or 90 min in a THERMOmax microplate reader (Molecular Devices) with monitoring of cell density at OD_{650}, followed by determination of *agr* activation by the β-lactamase/nitrocefin assay (*18*). For inhibition assays, 80 μL of cells (9 × 10^8 cells /mL) was treated in duplicate with AIP analogues in the presence of the group-specific wild-type AIP agonist at a concentration of 100 nM, followed by the β-lactamase assay.

Data Analysis. The data from the β-lactamase assay are plotted as initial β-lactamase reaction velocity versus log peptide concentration. In some instances, the data are normalized to percent maximal activation for comparison of curves between assays. Individual agonist or antagonist concentration−response curves were fitted via nonlinear regression to the following four-parameter Hill equation, using PRISM 3.0 (GraphPad Software, San Diego, CA):

$$E = \text{basal} + \frac{E_{\max} - \text{basal}}{1 + 10^{(\log EC_{50} - \log[A])n_H}} \qquad (1)$$

where E denotes effect, [A] the agonist concentration, n_H the midpoint slope, EC_{50} the midpoint location parameter, and E_{max} and basal the upper and lower asymptotes, respectively. For inhibition curves, the midpoint location parameter from eq 1 was the IC_{50}, rather than the EC_{50}. All EC_{50} and IC_{50} values are listed in tabular format with a 95% confidence interval (CI), which represents the error associated with the measurements using each peptide stock solution.

RESULTS

Structural Independence of Tail and Macrocycle. Endocyclic hydrophobic residues in AIP-II appear to be important for receptor binding, whereas an exocylic residue (asparagine) is important for receptor activation. We asked whether these two regions of the peptide are structurally independent. To address this idea, two-dimensional NMR studies of AIP-II, trAIP-II, and alanine-substituted analogues were performed. Figure 2A shows a comparison of the chemical shift differences (CSDs) of the backbone amide protons of AIP-II alanine analogues (determined in 90% H_2O/10% D_2O, pH 4.0) relative to the native AIP-II. Substitution with alanine at any position slightly affects the CSDs surrounding the site of modification, without affecting other regions of the peptide backbone. The trend exhibited for the amide protons also holds true for the α and β protons (see Supporting Information), thus suggesting that the side chains in the tail of AIP-II do not influence the backbone and side-chain structure of the macrocycle and vice versa. This idea is further supported by the comparison between AIP-II and trAIP-II (performed in DMSO), which shows no significant CSDs between the two peptides in the five amino acids that are common between them (Figure 2B). In these experiments, we considered a CSD >0.1 Δppm as being significant. These results collectively suggest that the tail of AIP-II, containing the key residue at position 3, does not stably interact with the macrocycle in solution.

A similar chemical shift comparison between AIP-II and its lactam analogue (performed in DMSO) reveals dramatic CSDs (≫0.1 Δppm) in the amide, α, and β protons of residues within the macrocycle but not within residues of the tail (Figure 2B and Supporting Information), further suggesting that the tail and macrocycle are structurally independent. Furthermore, these results demonstrate that the lactam macrocycle has a very different structure in solution versus the thiolactone, which could account for the much weaker activity of the AIP-II lactam analogue for AgrC-II activation (*11*). A similar trend was observed by NMR when the peptides were dissolved in 90% H_2O/10% D_2O, pH 4.0, further supporting the inference of structural difference. Furthermore, chemical shift assignments (in DMSO) of the linear free acid version of AIP-II, previously reported as biologically inactive on AgrC-I, -II, and -III (*12*), reveals similarly dramatic CSDs in comparison to AIP-II, thus highlighting the necessity for the presence and appropriate structure of the AIP-II macrocyle for potent agonist activity (Figure 2B).

Structural independence was also examined between the macrocycle and tail of AIP-I and AIP-IV. Two-dimensional NMR was performed on full-length AIP-I and -IV and compared to their truncated analogues, trAIP-I and trAIP-IV (Table 1 and Supporting Information). The CSDs in the amide, α, and β protons were never any greater than 0.3 Δppm and were in most instances less than 0.1 Δppm. This outcome is similar to the case of AIP-II and trAIP-II, supporting the inference of structural independence. Furthermore, acetylation of the N-terminus of AIP-I (Table 1) only results in local CSDs within the tail, as compared to AIP-I, without causing significant CSDs in the macrocycle.

Role of the N- and C-Termini Using AIP-I. To probe the steric and electrostatic requirements of the N-terminus of AIP-I in terms of receptor binding, a series of N-terminally modified analogues were synthesized. We first prepared an N-acetylated AIP-I via solid-phase peptide synthesis. This derivative activates AgrC-I and inhibits AgrC-II and -III with values comparable to those obtained with native AIP-I (Table 2; unless otherwise listed, all activation and inhibition results are presented in the table, including 95% confidence intervals). N-Acetylated AIP-I does not activate or inhibit AgrC-IV at any concentration tested, which is consistent with the fact that AIP-I only weakly cross-activates AgrC-IV (Table 2). As a further test of the above hypothesis, relatively

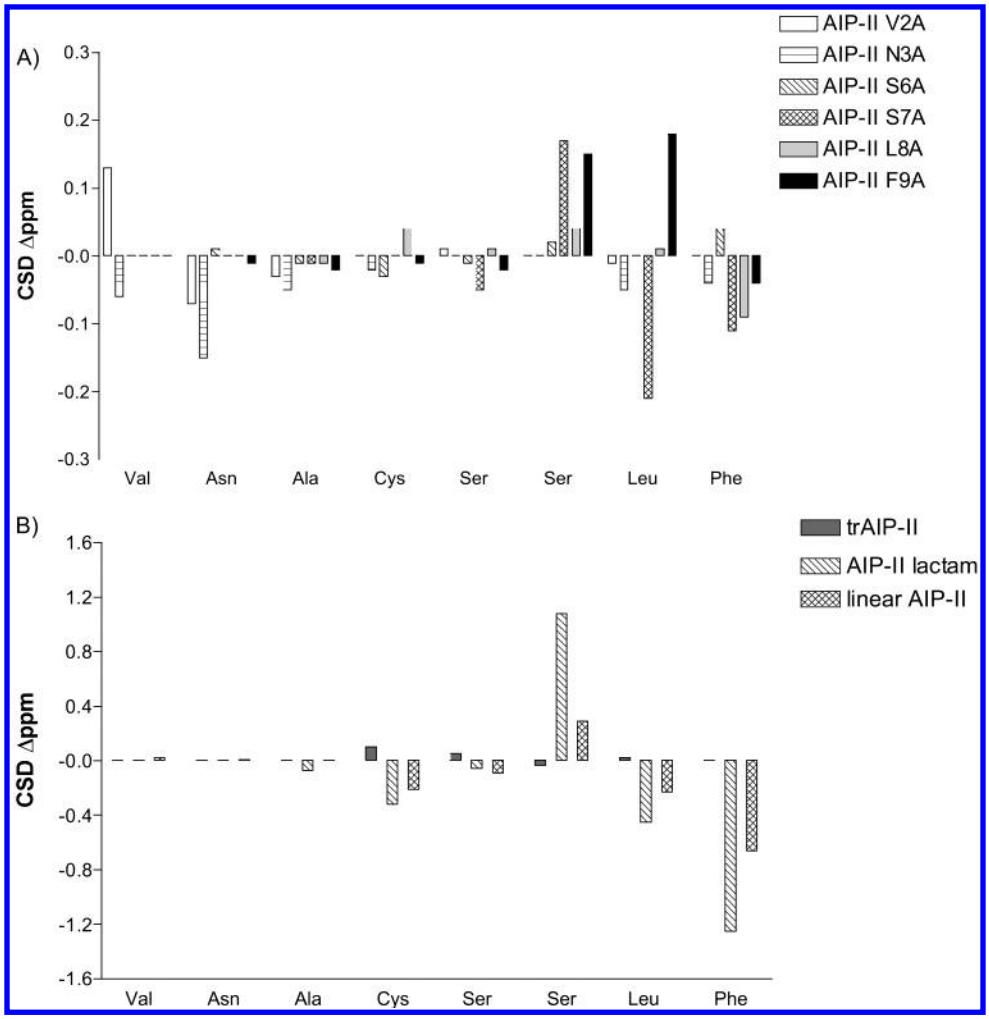

FIGURE 2: Chemical shift comparisons. (A) The amide ^{1}H chemical shifts of AIP-II alanine analogues are depicted as chemical shift differences (CSDs) relative to native AIP-II normalized to zero (performed in 90% H_2O/10% D_2O, pH 4.0 at 298 K). (B) The amide ^{1}H chemical shifts of trAIP-II thiolactone, the AIP-II lactam analogue, and the linear free acid version of AIP-II are depicted as chemical shift differences (CSDs) relative to native AIP-II normalized to zero (performed in DMSO-d_6 at 298 K).

bulky fluorescein or biotin groups were attached to the free amino terminus of AIP-I. These additions did not have any major effect on AIP-I function, as both fluorescein-conjugated AIP-I and biotinylated AIP-I could activate AgrC-I and inhibit AgrC-II at concentrations comparable to AIP-I in the nanomolar range, with an EC_{50} value for AgrC-I activation by fluorescein-conjugated AIP-I of 40 nM (22−74 nM, 95% CI). Thus, the function of AIP-I is not dramatically affected by acetylation or the addition of bulky groups on the N-terminus, suggesting that the ligand binding pocket can either accommodate these bulky groups or, more likely, that the N-terminus is solvent exposed.

In other studies, we tested whether the C-terminal hydrophobic amino acid, methionine, in AIP-I could be replaced by another hydrophobic amino acid without affecting function. Therefore, we synthesized AIP-I with isoleucine replacing methionine at position 8. AIP-I M8I activates AgrC-I as well as native AIP-I (Table 2) and similarly inhibits AgrC-II and -III, as compared to native AIP-I. AIP-I M8I activates AgrC-IV more effectively than does AIP-I (730 nM vs 26 μM), suggesting that this substitution permits improved binding and/or ligand-induced receptor activation by this analogue. Last, substitution of methionine with leucine in *agrD* by genetic methods results in a bacterial strain that autoinduces in a manner similar to that of wild-type cells (G. Ji and R. P. Novick, unpublished observation). These data collectively indicate that the thioether linkage in methionine is not required for agonist activity, which is inconsistent with a recent proposal from McDowell et al. that this linkage is critical (*14*).

Effect of Tail Length Using AIP-III. The chemical structures of various AIPs have been previously ascertained either by preparation of synthetic peptides with demonstrated activity or by peptide sequencing of AIPs purified from culture supernatants. The AIPs thus far examined by these methods include a heptapeptide from *Staphylococcus lugdunensis* (*7*), octapeptides from group I *S. aureus* (*7*, *12*), group IV *S. aureus* (see below), and *Staphylococcus epidermidis* (*13*), and a nonapeptide from group II *S. aureus* (*7*, *12*). We chose to verify the sequence of AIP-III using the former method of synthetic peptide preparation. On the basis of sequence similarities to AIP-I and the ability of AgrB from group I or III to process the group I or III AgrD propeptide in vivo, it was initially assumed that AIP-III would be an octapeptide (*7*). Accordingly, the octapeptide thiolactone was synthesized and shown to have cross-inhibitory activity toward group I cells. However, *agr* activation of AgrC-III could not be demonstrated with this peptide (*7*, *12*). Indeed, the thiolactone octapeptide turns out to be an antagonist of AgrC-III activation by group III *agr*+

Table 2: AIP Activity on the Four *agr* Groups of *S. aureus*

	activation (EC_{50})				inhibition (IC_{50})			
AIPs	AgrC-I	AgrC-II	AgrC-III	AgrC-IV	AgrC-I	AgrC-II	AgrC-III	AgrC-IV
AIP-I	28 nM (18−46)	−	−	26 μM (23−29 μM)	−	25 nM (14−45)	3 nM (2−5)	−
AIP-I D5N	90 nM (60−130)	−	360 nM (280−450)	−	−	32 nM (20−50)	−	20 nM (15−30)
AIP-IV	62 nM (52−75)	−	−	13 nM (7−40)	−	4 nM (3−5)	1 nM (0.5−3)	−
AIP-IV Y5F	10 nM (1.5−30)	−	−	14 nM (7−24)	−	2 nM (1.5−3)	0.2 nM (0.05−0.3)	−
AIP-I D5A	−	−	−	−	5 nM (3−7)	8 nM (4−17)	0.3 nM (0.2−0.4)	3 nM (2−5)
AIP-I M8I	30 nM (20−43)	−	−	730 nM (640−820)	−	13 nM (8−20)	8 nM (7−9)	−
Ac-AIP-I	98 nM (56−173)	−	−	−	−	60 nM (50−70)	12 nM (8−18)	−
trAIP-I	5.8 μM (3−10 μM)	−	−	−	4.5 μM (3.5−6 μM)	47 nM (40−55)	240 nM (40−1200)	−
trAIP-IV	−	−	−	−	930 nM (570−1521)	8 nM (6−10)	10 nM (5−22)	4.1 μM (3−5.5 μM)
trAIP-I D2A	−	−	−	−	5 nM (3−7)	5 nM (3−8)	0.1 nM (0.03−0.2)	5 nM (4−6)
AIP-II	−	30 nM (10−90)	−	−	40 nM (12−140)	−	1 nM (0.7−2.6)	86 nM (65−111)
trAIP-II	−	−	−	−	260 nM (95−695)	230 nM (190−270)	4 nM (3−5)	150 nM (90−260)
AIP-II/I	−	−	−	−	510 nM (360−810)	70 nM (33−147)	350 nM (290−420)	9.9 μM (6−16 μM)
AIP-I/II	−	−	−	−	10 nM (6−15)	230 nM (140−370)	0.3 nM (0.2−0.4)	12 nM (9−15)
AIP-III	−	−	26 nM (22−31)	−	70 nM (30−150)	6 nM (5−6.5)	−	150 nM (104−207)

[a] The dash (−) indicates no activity at any concentration tested up to at least 10 μM.

supernatant, with an IC_{50} of 360 nM (300−430 nM, 95% CI) (Figure 3A). Therefore, the octapeptide is clearly not the native AIP-III. The group III thiolactone hepta- and nonapeptides were synthesized according to the group III *agrD* amino acid sequence. The nonapeptide does not activate AgrC-III up to a concentration of 2.6 μM and instead inhibits AgrC-III activation by group III *agr*+ supernatant, with an IC_{50} of 790 nM (480−1310 nM, 95% CI). On the other hand, the thiolactone heptapeptide is an activator of AgrC-III, with an EC_{50} of 26 nM (Figure 3B, Table 2). Furthermore, no additive or inhibitory effects were observed when the heptapeptide was added with group III *agr*+ supernatant to the group III cells, with the synthetic peptide at various concentrations up to 60 μM. This suggests that the heptapeptide is likely to be the native AIP-III.

Further analysis supports this hypothesis, as the heptapeptide mimics what has been seen with group III *agr*+ supernatants, as well as with supernatants from other groups (*7*). It does not activate AgrC-I, -II, or -IV at any concentration tested up to 30 μM and instead inhibits their activation (Table 2). Furthermore, AIP-I, -II, and -IV inhibit AgrC-III activation by the heptapeptide (Table 2). These data demonstrate that the thiolactone heptapeptide corresponds to the native AIP-III, which is further supported by tandem mass spectrometry analysis performed on group III culture supernatants indicating the presence of the heptapeptide (M. Kalkum and B. Chait, unpublished observations). The fact that the addition of amino acids onto the N-terminus of AIP-III abolishes activation of AgrC-III but that the addition of functional groups onto the N-terminus of AIP-I has little effect on its activity indicates that the N-terminus plays different functional roles in the AIPs thus far examined.

Activity of AIP-IV. Several laboratories have previously identified a new group of *S. aureus* strains, designated group IV (*6*, *14*, *19*). Group IV *agr*+ supernatant activates RNAIII expression in group IV strains and the P3 promoter in a group IV reporter strain, while inhibiting *agr* expression in group II and III strains. Furthermore, group IV *agr*+ supernatant activates group I cells (*6*) and also activates to a similar extent a chimeric receptor, in which the sensor domain from AgrC-I is fused to the HK domain from AgrC-IV (*11*). Such data indicate that AIP-IV is a cross-activator of AgrC-I. However, it has recently been reported that the synthetic octapeptide AIP-IV is an inhibitor of group I *agr* expression, with an IC_{50} of 7 μM, rather than a cross-activator (*14*). McDowell et al. suggested that the difference in activity between the synthetic AIP-IV and bacterial supernatants could be accounted for by the presence in the supernatant of other components capable of influencing *agr* expression. As there is no other known extracellular signal that has been reproducibly confirmed to activate the *agr* system (*20*), it would be important to confirm the existence of such a factor, if it does exist. To test if another factor is required for activation, the octapeptide thiolactone AIP-IV was synthesized and assayed in the absence of group IV culture supernatant.

In our hands, synthetic AIP-IV is an activator of AgrC-IV and -I, with EC_{50} values in the nanomolar range (Table 2). Our synthetic AIP-IV is, as expected, an antagonist of AgrC-II and -III activation (Table 2). Conversely, both AIP-II and AIP-III inhibit AgrC-IV activation by AIP-IV (Table 2). The above results mimic the activity previously seen with group IV *agr*+ supernatant (*6*, *11*), providing strong evidence

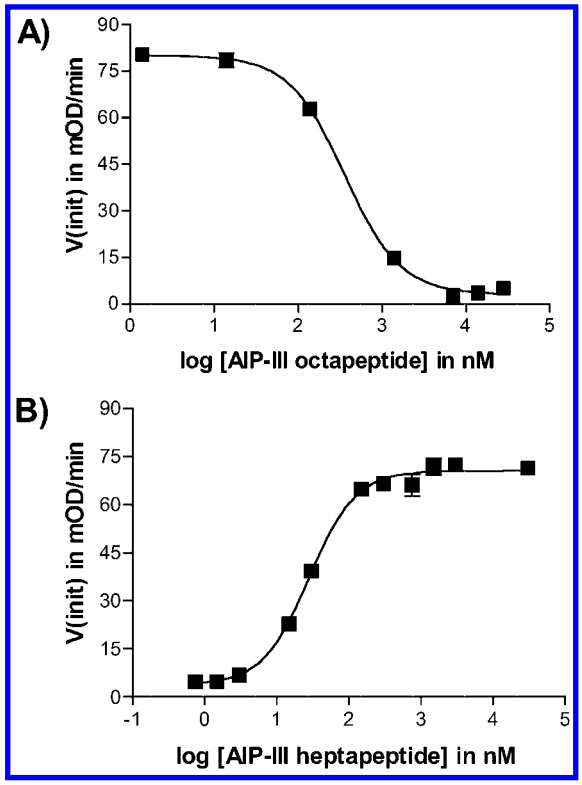

FIGURE 3: AIP-III studies. (A) Group III cells were incubated with a constant amount of group III culture supernatant with varying [AIP-III octapeptide]. (B) Group III cells were incubated with varying [AIP-III heptapeptide]. Data were collected as β-lactamase activity (V_{init} in mOD/min) and are shown at each concentration ± SEM. Unless otherwise visible, error bars are contained within the confines of the symbol.

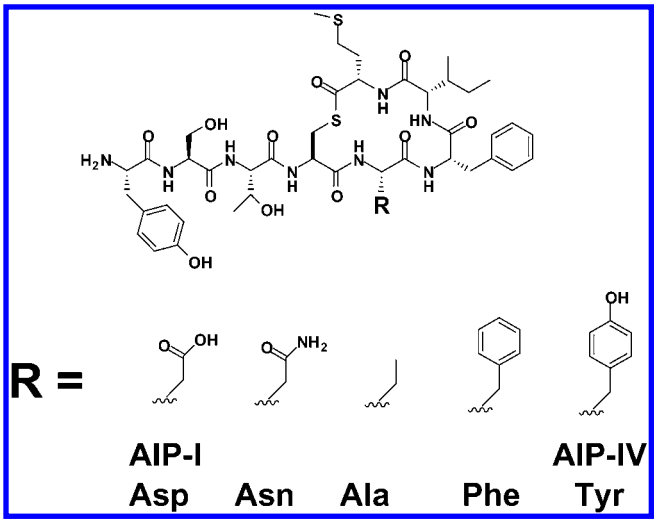

FIGURE 4: Structure−activity relationship studies on AIP-I and -IV. The chemical structure of the invariant portion of AIP-I and -IV is depicted, with an R group representing the site of modification. Various chemical groups were introduced at this position to probe different aspects of AIP-I and -IV function.

that the octapeptide thiolactone synthesized herein corresponds to the native AIP-IV. This is also consistent with tandem mass spectrometry analysis performed on group IV culture supernatants, which has indicated the presence of the octapeptide (M. Kalkum and B. Chait, unpublished observations). Since in our hands the activity of the synthetic AIP-IV mimics that of the culture supernatants, this strongly suggests that there is no additional factor in group IV culture supernatants which can activate group I cells and confirms that AIP-IV can cross-activate AgrC-I. It is noteworthy that McDowell et al. did not report whether their synthetic AIP-IV activates group IV cells (*14*).

Probing the Critical Side-Chain Differences between AIP-I and -IV. AIP-I and AIP-IV differ by only one amino acid: an endocyclic aspartate versus tyrosine in AIP-I and -IV, respectively (Figure 1). As shown above, the amino acid at this position profoundly affects the properties of the peptide, as AIP-I strongly activates AgrC-I but only weakly activates AgrC-IV, whereas AIP-IV strongly activates both. This contrasts with AIP-II, where substitution of this position by alanine (AIP-II S6A) did not result in any major change in bioactivity (*12*). This prompted us to begin structure−activity relationship studies of this critical residue in AIP-I and -IV to determine what role it plays in ligand−receptor binding and/or activation (Figure 4, Table 2). Initially, this residue was replaced with alanine. This peptide, abbreviated AIP-I D5A, does not activate AgrC-I or -IV at any concentration tested (up to 10 μM) and, instead, potently inhibits activation of both receptors (Table 2). The inhibition of AgrC-I by this analogue is consistent with the results on group I cells obtained by McDowell et al. using the same analogue (*14*). AIP-I D5A is also as potent as AIP-I and -IV in inhibiting AgrC-II and -III (Table 2). Thus, replacing the relatively bulky side chain at this position with an alanine methyl group has little to no effect on receptor binding but switches the peptide from an agonist into an antagonist.

To further probe the role of this position, more subtle modifications were introduced into AIP-I and AIP-IV. Substitution of the AIP-IV tyrosine with phenylalanine (Y5F) had no effect, as the peptide was similar to native AIP-IV in its activation/inhibition activities (Table 2), suggesting that the polar hydroxyl in tyrosine at position 5 is not required for bioactivity. In the case of AIP-I, the aspartate side chain is deprotonated at physiological pH, thus generating a negative charge at this position. To test whether a negative charge is required, we introduced an asparagine residue at this position. AIP-I D5N activates AgrC-I with an EC_{50} value only slightly higher than that of AIP-I (Table 2), thus demonstrating that the negative charge of the aspartate side chain is not essential for AgrC-I activation. AIP-I D5N does not activate AgrC-IV at any concentration up to 50 μM. Instead, AIP-I D5N inhibits AgrC-IV activation and also inhibits AgrC-II activation (Table 2). Remarkably, AIP-I D5N does not inhibit AgrC-III activation by AIP-III but rather activates group III cells, with an EC_{50} of 360 nM (Table 2). This example is the only case to date of conversion of an inhibitory AIP into an activator. All other previous substitutions have either inactivated the peptides or converted them from activators into inhibitors.

We tested if modification of the side chains of AIP-I and AIP-IV could affect the structure of the macrocycle, which might alter biological activity. Two-dimensional NMR chemical shift assignments confirmed the presence of the correct substitutions and demonstrated that the amide and α protons of AIP-IV Y5F and AIP-I D5N were not significantly different from those determined for AIP-IV and AIP-I, respectively (<0.1 Δppm differences at all positions). In the case of AIP-I D5A, comparison of the chemical shifts to those of AIP-I revealed no significant differences, except at position 5, where local effects from the alanine substitution

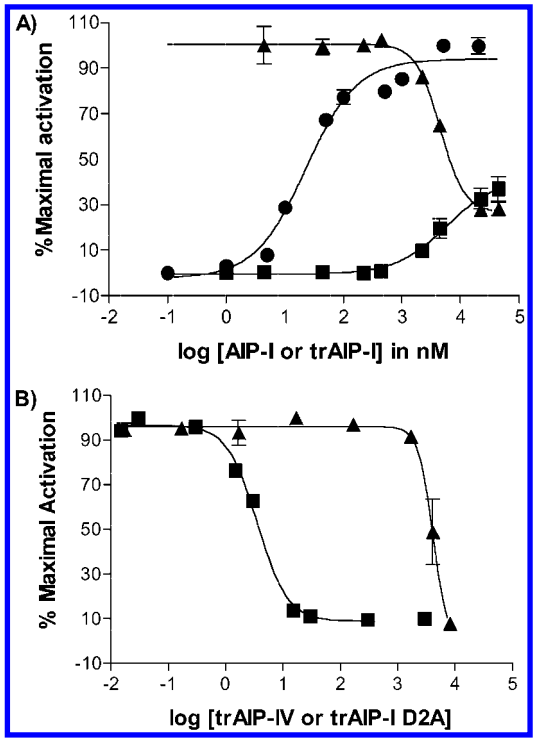

FIGURE 5: trAIP studies. (A) Group I cells were incubated with (■) varying [trAIP-I] alone or (▲) varying [trAIP-I] and a fixed concentration of AIP-I at 100 nM. The AIP-I concentration−response curve (●) is shown for comparison. (B) Group IV cells were co-incubated with (▲) varying [trAIP-IV] and a fixed concentration of AIP-IV at 100 nM or (■) [trAIP-I D2A] and a fixed concentration of AIP-IV at 100 nM. Data were collected as β-lactamase activity (V_{init} in mOD/min), normalized to % maximal activation, and are shown at each concentration ± SEM. Unless otherwise visible, error bars are contained within the confines of the symbol.

were observed. Thus, these substitutions have little effect on the backbone structures of the peptides in solution.

Properties of trAIP-I and trAIP-IV. The endocyclic position 5 in AIP-I and -IV is a key determinant for AgrC-I and -IV activation, respectively, whereas the exocyclic position 3 in AIP-II is a key determinant for AgrC-II activation (*11*). Removal of the tail of AIP-II and thus removal of position 3 converted AIP-II into an antagonist (*9*). Therefore, removal of the tail in AIP-I might produce a peptide retaining activating activity. Accordingly, an AIP-I derivative (trAIP-I) was synthesized, in which the tail was replaced by an acetyl group (Tables 1 and 2). This expectation seems to have been realized, at least in part, as this peptide by itself activates AgrC-I very weakly at high concentrations but also inhibits AgrC-I activation by AIP-I, with EC_{50} and IC_{50} values that are not significantly different from one another (Table 2). This concentration-dependent inhibition by trAIP-I reduces AgrC-I activation by AIP-I to the maximal activation observed with trAIP-I alone (Figure 5A). A ligand with decreased efficacy of activation is defined as a partial agonist (*21*), and it is, to our knowledge, the first time that such a ligand has been characterized in a bacterial signaling system.

TrAIP-I neither activates nor inhibits AgrC-IV at any concentration up to 44 μM, indicating that it most likely does not bind AgrC-IV. Therefore, the very weak activity of full-length AIP-I on group IV cells is abolished completely by removal of the tail. The fact that trAIP-I is a partial agonist only at very high concentrations for AgrC-I and no longer activates AgrC-IV at the highest concentration tested suggests that the tail contributes to receptor binding and/or activation in both instances but that the macrocycle itself can partially activate AgrC-I. TrAIP-I inhibits AgrC-II with similar potency to AIP-I (Table 2) and more weakly inhibits AgrC-III as compared to AIP-I (240 vs 3 nM). Therefore, removal of the tail has little effect on cross-inhibition of AgrC-II but does affect the potency of AgrC-III inhibition.

A truncated version of the AIP-1 D5A was also tested to determine whether the loss of activity by the substitution also resulted in a loss of partial agonism. This peptide, abbreviated trAIP-I D2A, has no detectable AgrC activation activity and is a strong inhibitor of activation of all four AgrC's (Table 2 and Figure 5B for group IV inhibition). This result underscores the key role of the amino acid at position 5 in AIP-I for AgrC-I activation.

Since the same critical activating residue occurs in AIP-IV, a truncated version of this peptide was synthesized and tested. Activation by trAIP-IV (Tables 1 and 2) was undetectable up to its solubility limit (~10 μM), and it was a weak, though complete, inhibitor of both AgrC-IV and -I, with IC_{50} values of 4.1 μM and 930 nM, respectively (Figure 5B, Table 2). Thus, partial agonism by trAIP-IV could not be detected and seems unlikely since it inhibits AgrC activation completely. Last, trAIP-IV is an inhibitor of AgrC-II and -III activation in the low nanomolar range (Table 2), suggesting that removal of the tail has little effect on cross-inhibition by trAIP-IV.

The partial agonism seen with trAIP-I again contrasts with a recent study, which reported that trAIP-I is solely an inhibitor of AgrC-I (*14*). This discrepancy can be explained by the fact that McDowell et al. utilized a cell line producing endogenous AIP-I for their bioassay, which might impede the observation of partial agonism by trAIP-I.

Properties of Chimeric AIPs. In an attempt to analyze further the remarkable differences between AIP-II and -I/-IV with respect to critical residues, we prepared chimeric peptides in which the tail of AIP-II was attached to the macrocycle of AIP-I and vice versa. If the macrocyle from AIP-I binds to AgrC-II in an orientation similar to that of AIP-II, then one might expect that this chimeric peptide, designated AIP-II/I (Table 1), might be able to activate AgrC-II. However, a different orientation of binding would most likely result in inhibition of AgrC-II activation. We tested this idea, which yielded the latter result, as AIP-II/I inhibits AgrC-II activation (Table 2). Furthermore, AIP-II/I also contains the key endocyclic residue for AgrC-I activation, aspartate, within its macrocyle, and the macrocyle alone (trAIP-I) is a partial agonist for AgrC-I. However, AIP-II/I does not activate AgrC-I at concentrations up to 20 μM and, instead, fully inhibits AgrC-I activation (Table 2). Therefore, addition of the tail from AIP-II to the macrocycle of AIP-I increases its inhibitory potency relative to trAIP-I and eliminates partial agonism by this analogue. Finally, AIP-II/I inhibited AgrC-III and -IV activation, with IC_{50} values of 350 nM and 9.9 μM, respectively, which compares to IC_{50} values of 240 nM and >44 μM for inhibition of AgrC-III and -IV by trAIP-I. This outcome suggests that addition of the AIP-II tail to the AIP-I macrocycle increases its affinity for binding to AgrC-IV while having no effect on binding to AgrC-III.

The reciprocal chimera, AIP-I/II, in which the tail from AIP-I was fused to the macrocyle of AIP-II (Table 1), inhibits AgrC-I, -II, -III, and -IV activation (Table 2). Comparing these IC_{50} values to those for trAIP-II (Table 2) suggests that the addition of the tail of AIP-I to the AIP-II macrocycle increases affinity for AgrC-I, -III, and -IV relative to trAIP-II without increasing the affinity for AgrC-II. The lack of activation by this analogue was expected, as it possesses neither of the residues from AIP-I or -II critical for activation.

Last, we utilized chemical shift mapping to determine whether the structure of the swapped macrocycles and tails in the chimeric AIPs changed as a result of fusing two different group-specific segments together. Two-dimensional NMR chemical shift assignments demonstrated that the amide and α protons of the macrocycles of AIP-I/II and AIP-II/I were not significantly different from those determined for AIP-II and AIP-I, respectively. In addition, the amide and α protons of the tails of AIP-I/II and AIP-II/I were not significantly different from those determined for AIP-I and AIP-II, respectively (see Supporting Information). This further supports the inference of structural independence of tail and macrocycle and suggests that the tails and macrocycles of the chimeric AIPs can assume structures similar to the segment of the native AIPs from which they are derived.

DISCUSSION

In this study, characterization of the AIPs from the four known *S. aureus agr* groups with respect to their activating and inhibiting activities has been completed. Several analogues of the AIPs synthesized in this study demonstrate that certain residues in the AIPs are critical for receptor activation. These key residues are not all located at corresponding positions, being endocyclic in some peptides and exocyclic in at least one (Figure 1). Furthermore, the interaction of these key residues with the receptor is critically dependent on the composition of the rest of the peptide. Moreover, the presence and composition of the tail exerts profound effects on the function of the AIP, even though it has little to no impact on the structure of the macrocycle, as determined by two-dimensional ^{1}H NMR. The structure of the macrocycle is also critically important for AIP function, as substitution of a lactam for the thiolactone, which has dramatic effects on the structure of the macrocycle, essentially eliminates agonist activity.

While AIP agonist activity is very sensitive to amino acid side-chain and structural changes, cross-inhibition by the AIPs seems to be more tolerant of sequence and structural diversity, as the AIP-II lactam and several alanine-substituted analogues have potent antagonist activity (*12*, *14*). This observation has led to the suggestion that cross-inhibition by the AIPs involves a binding interaction that is different from that of agonist AIP binding, although the inhibition is competitive (*11*) (illustrated schematically in Figure 6). This hypothesis is supported by experiments performed with chimeric AIPs, in which it was shown that a peptide possessing both of the residues in AIP-I and -II critical for agonist activity did not activate and instead universally inhibited AgrC activation (Table 2). Therefore, in the chimeric AIPs, these critical residues are not presented in the proper orientation within the ligand binding pocket of the receptor (Figure 6). This result provides a sharper focus on the general phenomenon of cross-inhibition by native AIPs, which usually differ from the group-specific AIP agonists at many positions. This means that particular residues in the tail and macrocycle must match in order for the AIP to bind in the correct orientation to activate the corresponding receptor; otherwise, competitive inhibition results.

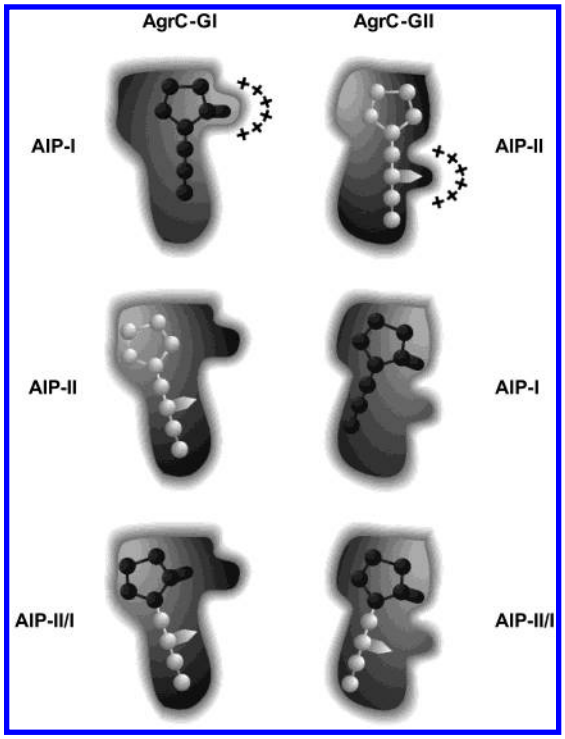

FIGURE 6: Model of receptor binding modes. AIP-I, AIP-II, and the chimeric peptide, AIP-II/I, are depicted schematically as bound to the ligand binding pocket of AgrC-I and -II. The key endocyclic residue (D5) of AIP-I and the key exocyclic residue (N3) of AIP-II insert into hypothetical grooves of AgrC-I and -II, respectively, triggering receptor activation, as illustrated by +++. Cross-inhibition most likely involves different orientations of binding to the heterologous receptor, as depicted by the tilt of the ligands in the binding pockets of AgrC-I and -II. The chimeric peptide, AIP-II/I, does not activate and instead inhibits AgrC-I and -II because it also binds in a different orientation, thus preventing the key residues from activating their respective receptor. This is a highly schematic model for the sole purpose of illustrating different modes of binding, and there is currently no information available concerning the ligand binding pocket within the sensor domain of AgrC.

Two-dimensional NMR studies did not reveal any significant chemical shift differences in the backbone protons of the truncated analogues versus their respective full-length AIPs, suggesting that the backbone structure of each macrocycle is not dramatically altered upon removal of the tail. However, the large difference in bioactivity between these analogues, with EC_{50} values in the nanomolar range for agonists and substantially higher IC_{50} values for truncated antagonists (Table 2), suggests that removal of the tail usually, but not always, results in decreased affinity of the ligands for the receptors. In the case of AIP-I and -IV, affinity can be regained through modification of the ligand, since trAIP-I D2A inhibits receptor activation in the nanomolar range. Another way in which affinity can be restored is by adding the tail from a different AIP. As the heterologous tail does not materially affect the structure of the macrocycle, it is likely to exert its effect through increased interactions

with the receptor. For example, the chimeric peptide, AIP-I/II, inhibits AgrC-I, -III, and -IV activation with IC_{50} values that are substantially lower than those seen with trAIP-II. In addition, AIP-II/I inhibits AgrC-I at nanomolar concentrations, in contrast to the inhibition in the micromolar range by trAIP-I (Table 2). This is not the whole story, however, since trAIP-I and -IV are only marginally less potent as inhibitors of AgrC-II than are the corresponding native AIPs.

Structure−activity studies of the endocyclic residue at position 5 in AIP-I and -IV revealed several significant features of the peptide−receptor interaction. Substitution of asparagine for aspartate in AIP-I converted a weak activator of AgrC-IV to a potent inhibitor, suggesting that the negative charge of the aspartate residue in AIP-I cannot be readily accommodated in the binding pocket of AgrC-IV. In contrast, the electrically neutral asparagine binds with relative ease in the AIP-IV binding pocket, though this does not result in AgrC-IV activation. The mechanistic basis for weak activation by AIP-I and inhibition by AIP-I D5N will only be fully understood once the ligand binding pocket in AgrC-IV has been characterized.

Activation of AgrC-I by AIP-I, AIP-IV, AIP-I D5N, AIP-IV Y5F and inhibition by AIP-I D5A suggest that a single side-chain methyl group at position 5 (i.e., AIP-I D5A) is insufficient for AgrC-I activation but that bulkier groups will do, with the exception that positively charged side chains have not been tested. Furthermore, the partial agonism of AgrC-I by trAIP-I but not by trAIP-I D2A suggests that the key aspartate residue confers activating properties on the macrocycle in the absence of the tail. Last, in the course of testing the various analogues, we observed activation of AgrC-III by AIP-I D5N. This activation is surprising, as native AIP-I is a typical cross-inhibitor of AgrC-III. The specificity and mechanistic basis for group III *agr* activation by this subtly modified analogue will be the subject of future studies.

The addition of extra residues on the N-terminus of AIP-III switches it from an agonist into an antagonist, and the addition of an N-terminal glycine to the *S. epidermidis* AIP inactivates the peptide (*13*). In contrast, N-acetylation or the addition of relatively bulky groups (such as fluorescein or biotin) to the N-terminus of AIP-I does not affect its activity. These contrasting results suggest that the effect of N-terminal modification varies for different AIPs. Furthermore, the mechanistic basis by which the processing enzyme, AgrB, specifies the length of the N-terminal tail of the AIPs, relative to the conserved C-terminal (DE) motif in the AgrD propeptide, remains a mystery.

The AIPs from the four *agr* groups in *S. aureus* and other AIP analogues have provided much new information, which ultimately will be best understood through characterization of their respective receptors. Therefore, future studies will address the receptors themselves to understand further the basis for activation and competitive antagonism of receptor signaling by the AIPs.

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SUPPORTING INFORMATION AVAILABLE

One table providing all proton NMR chemical shift assignments. This material is available free of charge via the Internet at http://pub.acs.org.

REFERENCES

1. Falke, J. J., and Hazelbauer, G. L. (2001) *Trends Biochem. Sci. 26*, 257−265.
2. Novick, R. P., and Muir, T. W. (1999) *Curr. Opin. Microbiol. 2*, 40−45.
3. Yu, E. W., and Koshland, D. E., Jr. (2001) *Proc. Natl. Acad. Sci. U.S.A. 98*, 9517−9520.
4. Novick, R. P., Ross, H. F., Projan, S. J., Kornblum, J., Kreiswirth, B., and Moghazeh, S. (1993) *EMBO J. 12*, 3967−3975.
5. Otto, M., Sussmuth, R., Jung, G., and Gotz, F. (1998) *FEBS Lett. 424*, 89−94.
6. Jarraud, S., Lyon, G. J., Figueiredo, A. M., Gerard, L., Vandenesch, F., Etienne, J., Muir, T. W., and Novick, R. P. (2000) *J. Bacteriol. 182*, 6517−6522.
7. Ji, G., Beavis, R., and Novick, R. P. (1997) *Science 276*, 2027−2030.
8. Dufour, P., Jarraud, S., Vandenesch, F., Greenland, T., Novick, R. P., Bes, M., Etienne, J., and Lina, G. (2002) *J. Bacteriol. 184*, 1180−1186.
9. Lyon, G. J., Mayville, P., Muir, T. W., and Novick, R. P. (2000) *Proc. Natl. Acad. Sci. U.S.A. 97*, 13330−13335.
10. Lina, G., Jarraud, S., Ji, G., Greenland, T., Pedraza, A., Etienne, J., Novick, R. P., and Vandenesch, F. (1998) *Mol. Microbiol. 28*, 655−662.
11. Lyon, G. J., Wright, J. S., Christopoulos, A., Novick, R. P., and Muir, T. W. (2002) *J. Biol. Chem. 277*, 6247−6253.
12. Mayville, P., Ji, G., Beavis, R., Yang, H., Goger, M., Novick, R. P., and Muir, T. W. (1999) *Proc. Natl. Acad. Sci. U.S.A. 96*, 1218−1223.
13. Otto, M., Sussmuth, R., Vuong, C., Jung, G., and Gotz, F. (1999) *FEBS Lett. 450*, 257−262.
14. McDowell, P., Affas, Z., Reynolds, C., Holden, M. T. G., Wood, S. J., Saint, S., Cockayne, A., Hill, P. J., Dodd, C. E. R., Bycroft, B. W., Chan, W. C., and Williams, P. (2001) *Mol. Microbiol. 41*, 503−512.
15. Novick, R. P. (1991) *Methods Enzymol. 204*, 587−636.
16. Bax, A., Byrd, R. A., and Aszalos, A. J. (1984) *J. Am. Chem. Soc. 106*, 7632−7633.
17. Kessler, H., Griesinger, C., Kerssebaum, R., and Wagner, K. (1987) *J. Am. Chem. Soc. 109*, 607−609.
18. Ji, G., Beavis, R. C., and Novick, R. P. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 12055−12059.
19. Otto, M., Echner, H., Voelter, W., and Gotz, F. (2001) *Infect. Immun. 69*, 1957−1960.
20. Novick, R. P., Ross, H. F., Figueiredo, A. M. S., Abramochkin, G., and Muir, T. (2000) *Science 287*, 391a.
21. Pliska, V. (1999) *J. Recept. Signal Transduction Res. 19*, 597−629.

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