Cysteine is the main precursor of sulfur-containing biological molecules in bacteria and contributes to the control of the cell redox state. Hence, this amino acid plays an essential role in microbial survival and pathogenicity and the reductive sulfate assimilation pathway is considered a promising target for the development of new antibacterials. Serine acetyltransferase (SAT) and O-acetylserine sulfhydrylase (OASS-A), the enzymes catalyzing the last two steps of cysteine biosynthesis, engage in the formation of the cysteine synthase (CS) complex. The interaction between SAT and OASS-A finely tunes cysteine homeostasis, and the development of inhibitors targeting either protein–protein interaction or the single enzymes represents an attractive strategy to undermine bacterial viability.

Given the peculiar mode of interaction between SAT and OASS-A, which exploits the insertion of SAT C-terminal sequence into OASS-A active site, we tested whether a recently developed competitive inhibitor of OASS-A exhibited any effect on the CS stability. Through surface plasmon resonance spectroscopy, we (i) determined the equilibrium constant for the Salmonella Typhimurium CS complex formation and (ii) demonstrated that the inhibitor targeting OASS-A active site affects CS complex formation. For comparison, the Escherichia coli CS complex was also investigated, with the aim of testing the potential broad-spectrum activity of the candidate antimicrobial compound.

Keywords: cysteine synthase; cysteine biosynthesis; protein–protein interactions; competitive inhibitor; antibacterial; surface plasmon resonance; fluorescence

1. Introduction

Sulfur metabolism in bacteria supports the maintenance of intracellular redox homeostasis and takes part in the prevention of oxidative stress damages. Low molecular weight thiols as glutathione, mycothiol, bacillithiol, and coenzyme A efficiently buffer the reducing environment and are generally present at high concentrations to accomplish their function. Their synthesis primarily depends on the reservoir of cysteine which, in turn, can be stored as low molecular weight thiols. Indeed, cysteine is prone to autoxidation generating toxic reactive oxygen species, and the control of its biosynthesis and free concentration inside the cell is essential for bacterial fitness. The protection from oxidative stress is strictly related to the bacterial survival to host defenses and during latency stages, as well as to the contrast of antibiotics action [1–4]. Indeed, pathogen strains auxotrophic for cysteine were found to be more susceptible to antibiotic administration being more exposed to oxidative...
stress damages [5–8], which represents a common mechanism of action of large spectrum antibiotics. Therefore, in the compelling quest for effective antimicrobials able to counteract the ever-growing insurgence of resistances, cysteine biosynthesis has been individuated as a promising nonessential target for the development of antibiotic enhancers [9–13].

Most bacteria rely on the well-conserved reductive sulfate assimilation pathway for cysteine biosynthesis. The last two steps are catalyzed by serine acetyltransferase (SAT, EC 2.3.1.30) and O-acetylserine sulfhydrylase A (OASS-A, EC 2.5.1.47). SAT, a homo-hexameric enzyme, synthetizes O-acetylserine (OAS) in the presence of L-serine and acetyl coenzyme A, with OAS subsequently converted to L-cysteine by an OASS-A dimer in the presence of bisulphide. SAT and OASS-A engage in the formation of the multienzymatic complex cysteine synthase (CS), where a C-terminal tail of one subunit in each SAT trimer binds to one OASS-A active site and stabilizes the assembly, resulting in a 3:2 stoichiometry [14–18]. The activity of SAT is maximized when bound to OASS-A, while the latter is inhibited [17], although only a part of total OASS-A in the cell is involved in CS formation [19]. Even though the physiological function of CS has been only hypothetically assessed [20–22], it is well-established that its equilibrium is sensitive to the concentration of several metabolites, resulting in a fine-tuning of the two enzymatic activities. In fact, high concentrations of bisulphide stabilize CS complex [14], whereas OAS promotes its dissociation [14,23–25]; on the other hand, increasing levels of cysteine can inhibit SAT catalytic activity by feedback control, triggered by binding to its active site [26,27]. The alteration of this metabolic equilibrium can induce profound consequences in bacterial fitness, with several potential pleiotropic effects involving the numerous moonlighting functions of OASS-A [28–31].

In the last years, many efforts have been addressed to the development of potential inhibitors targeting cysteine pathway [32–34] and, in particular, OASS isoforms or SAT from several pathogen species [10,35–48]. A drug discovery campaign carried out in our laboratories led to the identification of the synthetic compound UPAR415 ((1S,2S)-1-(4-methylbenzyl)-2-phenylcyclopropanecarboxylic acid, Figure 1a) as the most potent inhibitor developed so far of OASS-A from Salmonella Typhimurium (StOASS-A), with a nanomolar dissociation constant for the target [41,49]. Microbiologic assays demonstrated a synergic and/or additive action of the compound with colistin, a last-line polycationic antibiotic, in several bacterial strains prone to the insurgence of antibiotic resistance. Target engagement experiments on S. Typhimurium cultures and cytototoxicity assays on eukaryotic cells, moreover, determined the inhibitor to be specific and nontoxic [49]. These data support the viability of the pursued approach and represent a good starting point for the development of a new class of antimicrobial compounds, targeting the enzymes of the sulfur reductive assimilation pathway. UPAR415, identified after several rounds of modification and selection of substituted cyclopropanes, is inspired to the C-terminal isoleucine of SAT, an extremely well-conserved residue responsible for CS anchorage [24,50]; because of its structure, this compound might destabilize the CS complex by competing with SAT tails for the occupation of OASS-A active site. Very recently, the pose of UPAR415 bound to StOASS-A was confirmed through crystallization studies (Figure 1, Protein Data Bank (PDB) id 6z4n, [49]). With this in mind, we decided to extend the mechanistic characterization of UPAR415 to a broader biochemical context, involving the engagement of OASS-A in the CS complex. The formation of the CS complex of S. Typhimurium has been herein investigated through surface plasmon resonance (SPR). The affinity between OASS-A and SAT, previously measured under conditions that partially destabilize the complex [51–53], was determined and found to be similar to that of the homologous complex from other Gammaproteobacteria, such as Escherichia coli and Haemophilus influenzae. The effects of UPAR415 on the formation of CS complex were then examined by exploiting SPR and static fluorescence experiments. Given the high sequence and structural homology of the constituent enzymes, the investigation was extended to the CS complex from E. coli, another emerging antibiotic-resistant bacterium for which innovative treatments are urgently required.
2. Results and Discussion

2.1. Determination of the Affinity of Salmonella Typhimurium Cysteine Synthase Complex

The formation of a cysteine synthase complex between OASS-A and SAT in S. Typhimurium and E. coli was first reported by Kredich and colleagues in the late 1960s [14,19,54,55], when serine transacylase and O-acetylserine sulfhydrylase activities were co-purified, casting questions about the assembly composition, function, and dynamics. In the following decades, CS complexes were identified in other organisms, including bacteria, protozoa, and plants [16,56–59]. In this context, the engagement and the affinity of OASS-A and SAT in bacteria have been the object of several biophysical and kinetic studies, because of the potential pharmaceutical relevance of the cysteine biosynthetic pathway [15,17,24,31,52,60–63]. Studies on S. Typhimurium and E. coli are of particular relevance because of the inclusion of these two species in the global priority list of the World Health Organization for the insurgence of new antibiotic resistants [64].

Many studies describe the function and regulation of OASS-A and SAT from E. coli (EcOASS-A and EcSAT, respectively). Moreover, pre-steady state and SPR experiments allowed the determination of E. coli CS (EcCS) dissociation constant, found to be in the low nanomolar range [24,62]. On the other hand, despite the pioneering studies that led to the original discovery of the complex and the wealth of information available about the single proteins, the CS complex from S. Typhimurium (StCS) has been poorly investigated and several considerations reported in the literature are deduced by the high degree of similarity to the E. coli proteins (sequences identity >95%). Among the available data, SPR experiments carried out by Kaushik and colleagues on StCS indicate a $K_D$ of 30–70 nM, also supported by other biophysical approaches [53]. This would indicate an affinity about an order of magnitude lower than that observed for the E. coli complex. These results, however, are likely affected by the experimental conditions: indeed, the chloride ions present in the environment.
buffer at high concentration promote the transition of OASS-A to an inhibited conformation (PDB id 1fcj, [51]), thus affecting the formation of the complex [52].

To measure the affinity of the StCS complex by SPR, we exploited the immobilization of OASS-A by amine coupling on a CM5 golden chip. Increasing concentrations of StSAT (in the range of 0.5–50 nM) were sequentially injected and flushed on the chip at 25 °C to obtain a single-cycle kinetic sensorgram (Figure 2a). The data were fitted to the Langmuir 1:1 equation (see Materials and Methods), the simplest binding model, giving an equilibrium $K_D$ for the complex formation of $2.3 \pm 0.6$ nM. To validate our approach, the same experimental setup was applied for the determination of the affinity of the *E. coli* complex (Figure 3a). The calculated affinity at the equilibrium was $2.3 \pm 0.1$ nM, in perfect agreement with the published data reported by other groups [24, 62]. The analogy of the two constants, moreover, is in accordance with the high sequence and structural conservation of the partner proteins from *S. Typhimurium* and *E. coli*, and with the comparable tertiary conformations stabilized upon CS formations, that appears to favor an open-to-close rearrangement of both OASS-A subunits [18, 65].

### 2.2. Interaction of UPAR415 and SAT with OASS-A

The structure of StOASS-A in complex with UPAR415 has been recently solved. The molecule accommodates in the enzyme active site and occupies, through its carboxylate moiety, the anchoring subsite of the C-terminal tail of SAT (Figure 1b, [16, 49]). As demonstrated by the superimposition with the structure of *H. influenzae* OASS-A (HiOASS-A) bound to the SAT C-terminal decapeptide (PDB id 1y7l, [16]) or pentapeptides [36], the pose of UPAR415 mimics that of the C-terminal isoleucine residue. Due to the steric hindrance of the tolyl substituent, an evident closure movement is not elicited and OASS-A maintains a conformation similar to the one observed in the absence of ligands (PDB id 1oas, [65]). This narrow rearrangement also manifests upon peptides binding to HiOASS-A, which is unable to induce the large conformational changes that are assumed to take place upon full-length SAT binding, leading the transition to the closed conformation [18]; moreover, the peptides bind with much lower affinity to OASS-A (in the low micromolar range), supporting the existence of a more extended interaction network between SAT and OASS-A [36]. The indirect evidence of the different structural rearrangement upon binding of StSAT or UPAR415 is provided by static fluorescence measurements. OASS-A is a pyridoxal-5′-phosphate (PLP)-dependent enzyme and the cofactor can be exploited as an intrinsic probe to monitor the ligand binding to the active site of the enzyme, by changes in fluorescence intensity and emission wavelength [15, 36, 50, 66]. Upon direct excitation of PLP at 412 nm, the emission signal of StOASS-A at 505 nm, in the absence of ligands, shifts to 502 nm in the presence of UPAR415 and 495 nm in the presence of StSAT, respectively (Figure 4a). These blue shifts may indicate the formation of a less polar environment within the active site, possibly associated with a closure or a rearrangement of the pocket leading to water exclusion, which is more pronounced in the case of SAT binding. The same emission behavior is displayed by EcOASS-A, and based on structural data, a comparable conformational change can be hypothesized (Figure 4b).
Figure 2. Kinetics of formation of StCS complex in the absence (a) and in the presence of 0.15 µM (b), 0.3 µM (c), 1 µM (d) or 2 µM (e) UPAR415. Data were fitted to the Langmuir 1:1 binding model (red lines). SAT was injected at five sequential concentrations of 0.5, 2.5, 5, 25, and 50 nM (a–c) or 2.5, 5, 25, 50, and 100 nM (d,e). Protein concentrations are expressed as monomers.
Figure 3. Kinetics of formation of EcCS complex in the absence (a) and in the presence of 0.15 µM (b), 0.3 µM (c), 1 µM (d) or 2 µM (e) UPAR415. Data were fitted to the Langmuir 1:1 binding model (red lines). SAT was injected at five sequential concentrations of 0.5, 2.5, 5, 25, and 50 nM (a–c) or 2.5, 5, 25, 50, and 100 nM (d,e). Protein concentrations are expressed as monomers.
Changes in fluorescence emission spectra (λex = 412 nm) of StOASS-A (a) or EcOASS-A (b) in the absence (black) and in the presence of saturating SAT (purple) or UPAR415 (pink). The peak is centered at 495 nm when CSC is formed and at 502 nm when the inhibitor is bound to OASS-A.

The fluorescence quantum yield increase upon UPAR415 binding was followed to determine the binding affinity for OASS-A. Previous studies reported the KD of UPAR415 for StOASS-A at 20 °C (28 ± 5 nM, [41]); as a comparison, the constant for EcOASS-A was determined in the same experimental conditions (KD = 48.3 ± 6.2 nM, data not shown) and was found to be in agreement with the values reported for the S. Typhimurium isoform. Because of the different temperature at which the SPR measurements have been carried out and the lack of binding experiments on EcOASS-A, we first determined the dissociation constants of UPAR415 for the two OASS-A orthologs at 25 °C (Figure 5). The calculated values for KDs are 97.3 ± 6.3 nM and 55.8 ± 8.0 nM for the S. Typhimurium and the E. coli enzymes, respectively, indicating that the molecule, developed for StOASS-A inhibition, binds the E. coli isoform with a very similar affinity.

Binding of UPAR415 to StOASS-A (a) and EcOASS-A (b) at 25 °C. Fluorescence emission intensities at 500 nm upon excitation at 412 nm were fitted to a tight-binding equation (Equation (5)) giving KD values of 97.3 ± 6.3 nM for StOASS-A and 55.8 ± 8.0 nM for EcOASS-A.
2.3. Effects of UPAR415 on CS Complex Formation

Following the same strategy exploited for the determination of the CS complex affinity, we moved to the SPR analysis of the effect of UPAR415 on the formation of the CS complex of both S. Typhimurium and E. coli. OASS-A was immobilized on the sensor chips and single-cycle kinetics were run by injecting increasing concentrations of SAT in the presence of a fixed concentration of UPAR415 per experiment (Figures 2b–e and 3b–e). These measurements allowed us to determine the CS complexes affinity decrease as induced by the inhibitor (Table 1). SAT and UPAR415 compete for the binding to OASS-A active site, and their concurrent presence reciprocally influences their apparent affinities for the target. Sensorgrams mirror the kinetics of SAT:OASS-A interaction, whose fitting to the Langmuir 1:1 binding model provides the apparent dissociation constant of CS complex as influenced by the different concentrations of UPAR415. Fitting of the sensorgrams reported in Figures 2 and 3 can also provide information on the kinetic parameters for complex formation and dissociation and how these are eventually influenced by UPAR415. However, CS formation, as reported for other bacterial species [31,52], is expected to be a complex, multistep process, and its accurate kinetic description is out of the scope of the present work.

Table 1. Comparison between experimental $K_D$ of CS complex (CSC) from S. Typhimurium and E. coli in the presence of UPAR415 and predicted values obtained by Equation (4) (see Materials and Methods). The $K_D$ values obtained by SPR measurements in the absence of inhibitor were used to calculate the predicted apparent dissociation constants. Errors are expressed as standard deviation.

| [UPAR415] (µM) | Technique | Experimental $K_{D,app}$ CSC (nM) | Predicted $K_{D,app}$ CSC (nM) |
|----------------|-----------|----------------------------------|-------------------------------|
|                |           | S. Typhimurium | E. coli | S. Typhimurium | E. coli |
| 0              | SPR       | 2.3 ± 0.6      | 2.3 ± 0.1 | -             | -      |
| 0.15           | SPR       | 2.7 ± 0.8      | 2.8 ± 0.1 | 3.4           | 3.9    |
| 0.3            | SPR       | 19.4 ± 2.4     | 6.0 ± 0.4 | 4.5           | 5.5    |
| 1              | SPR       | 20.3 ± 0.7     | 11.7 ± 0.9 | 9.7           | 13.0   |
| 2              | SPR       | 37.0 ± 3.9     | 35.6 ± 3.1 | 17.0          | 23.7   |
| 2              | Fluorescence | 59.2 ± 4.2 | 69.1 ± 8.6 | 17.0          | 23.7   |
| 5              | Fluorescence | 125.5 ± 19.3 | 123.5 ± 39.4 | 39.1         | 55.8   |
| 10             | Fluorescence | 253.7 ± 50.8 | 269.2 ± 53.5 | 75.9         | 109.3  |

Concentrations of inhibitor above 2 µM hampered the derivation of reliable SPR data since the apparent strength of the complexes is very likely out of the range of instrumental sensitivity (data not shown). We decided then to exploit static fluorescence experiments to investigate the behavior of the CS complexes at higher UPAR415 concentrations. Since the fluorescence quantum yield of the OASS-A:UPAR415 complex is similar to that obtained by the binding of SAT (Figure 4), the wavelength emission shifts were instead employed to monitor the displacement of the inhibitor from OASS-A active site by SAT. OASS-A was titrated with increasing concentrations of SAT in the presence of 2 µM, 5 µM or 10 µM inhibitor (Figure 6) at 25 °C. Wavelength maxima shifts from 502 nm (OASS-A:UPAR415 complex) to 495 nm (OASS-A:SAT complex) were plotted against the SAT concentration and apparent $K_D$ values were obtained by fitting data to a binding isotherm (Table 1). The 1:1 binding model applied to SPR data analysis allowed a direct comparison with static fluorescence data. Figure 7 reports the increase in the apparent dissociation constants of CS complex as a function of UPAR415 concentration. Lines represent the fitting of the data to Equation (4) (see Materials and Methods), rearranged to derive the apparent dissociation constant $K_{D,app}$. The intercept represents the $K_D$ for CS complex (CSC) in the absence of inhibitor, whereas the slope corresponds to $K_D$ CSC/$K_D$ U415 ratio. The linearity of the observed behavior confirms the complementarity of the data obtained through the two different approaches. The experimental point at 2 µM UPAR415 was calculated both by SPR and static fluorescence measurements, further demonstrating a very good overlap of the results.
As can be noticed from data reported in Table 1 and Figure 7, UPAR415 elicits superimposable effects on the formation of StCS and EcCS complexes. Based on the increment of the retrieved apparent K_{D,app} of CS complexes (K_{D,app CSC, Table 1}) in the presence of increasing concentrations of inhibitor, it is possible to observe that UPAR415 promotes the destabilization of the complexes formation by competing with SAT. Accordingly, when Equation (4) (see Materials and Methods), which assumes a simple competitive binding of two ligands for the same site, is applied to calculate the K_{D,app} from the K_{D}s of CS complexes in the absence of inhibitor, the theoretical values agree very well with the experimental results (Tables 1 and 2).
Table 2. CS complex dissociation constants obtained from the fitting with Equation (4) (Figure 7) of SPR and fluorescence data, corresponding to the $K_{D,\text{app}}$ values for CS complex formation in the presence of increasing concentrations of UPAR415.

| CS Complex       | $K_D$ CSC (nM) | $K_D$ CSC/$K_D$ UPAR415 (nM) | Extrapolated $K_D$ UPAR415 (nM) | $R^2$ |
|------------------|---------------|-----------------------------|---------------------------------|------|
| S. Typhimurium   | 0.83          | 0.025                       | 33.2                            | 0.99 |
| E. coli          | 0.63          | 0.025                       | 25.2                            | 0.95 |

As shown in Figure 7, the apparent $K_D$s were globally fitted to a linear equation (Equation (4)); the intercepts on the y-axes provided the theoretical affinity values for the formation of CS complex in the absence of inhibitor (0.83 nM for StCS and 0.63 nM for EcCS, Table 2), which are in agreement with the experimental data reported in Table 1. The ratios between the offsets on y-axes and the slopes of the fittings represent the $K_D$s of U415 for OASS-A (Table 2), giving a value of 33.2 nM for S. Typhimurium and 25.2 nM for E. coli, in good accordance with the $K_D$ values determined by fluorescence measurements with the direct titrations (Figure 5).

3. Materials and Methods

3.1. Chemicals and Reagents

All reagents were purchased from Merck (Darmstadt, Germany) and used as received, with the exception of acetyl coenzyme A, purchased from Applichem (Darmstadt, Germany).

3.2. Expression and Purification of Recombinant Proteins

Recombinant proteins were independently overexpressed in E. coli host in LB medium in the presence of selective antibiotics and, in the case of SAT, of 2% glucose to promote mild induction conditions and hinder cysteine operon induction by O-acetylserine (OAS) accumulation. Cultures were grown at 37 °C under shaking until 0.5–0.6 OD was reached; isopropyl β-d-1-thiogalactopyranoside was then added to the medium at 1 mM final concentration, and cells were let grown under shaking for four hours. Cells were harvested by centrifugation, washed with phosphate-buffered saline solution, and stored as pellets at −80 °C until further use. At the moment of purification, pellets were resuspended in lysis buffer (10 mL per gram of cells) in the presence of proteases inhibitors (1.5 µM pepstatin A, 0.2 mM benzamidine, 0.2 mM phenylmethanesulfonyl fluoride), 1 mg/mL lysozyme and, in the case of OASS-A, 0.2 mM PLP. Cells were sonicated in ice by 10 s on/1 min off cycles, for 4 min. The supernatant containing the soluble proteins was separated from the cellular debris by centrifuging the samples at 16,000 g for 30 min. All the proteins were purified on an ÄKTA Prime (GE Healthcare, Uppsala, Sweden) FPLC system.

SAT from S. Typhimurium (StSAT) and E. coli (EcSAT) was expressed in BL21 Tuner™ (DE3) cells and purified as constructs presenting a His$_6$-thioredoxin (TRX) tag at their N-termini, exploited to increase protein solubility and for IMAC purification on a Talon™ resin (Clontech Laboratories, Inc., Mountain View, CA, USA). Briefly, StSAT and EcSAT expressing cells were resuspended in buffer A (100 mM TRIS, 500 mM NaCl, 50 mM imidazole, 1 mM tricarboxyethyl phosphine (TCEP), 50 µM L-cysteine, pH 7.5), in the presence of protease inhibitors and sonicated as described above. The supernatants were loaded on cobalt resin, previously equilibrated in buffer A, and the column was additionally washed to remove aspecific proteins. A solution of 10 mM OAS in buffer A, pH 7.0, was then flushed to remove endogenous bacterial OASS-A possibly bound to recombinant SAT to avoid the presence of contaminants in the final preparation. StSAT and EcSAT were finally eluted in the presence of 500 mM imidazole and fractions containing His-tagged TRX-SAT were collected and put together in a 12 kDa-dialysis tube in the presence of 1 mM EDTA and 1 mM DTT. Then, 1 mg of in house produced His-tagged tobacco etch virus (TEV) protease every 50 mg of SAT was also added, to cut the His$_6$-TRX tag. The solutions were dialyzed O/N against 20 mM TRIS, 250 mM NaCl, 1% glycerol, pH 7.5, and the following day they were recovered, centrifuged, and loaded on the Talon™ resin to
remove His$_6$-TRX tags, uncleaved proteins, and TEV protease. The flow-through samples containing the purified SATs were then concentrated in an Amicon$^\text{®}$ stirred-cell (Merck-Millipore, Burlington, MA, USA), and the buffer was exchanged with 20 mM sodium phosphate, 250 mM NaCl, 2 mM EDTA, 10 mM 2-mercaptoethanol, pH 7.0. The proteins were loaded on a size-exclusion HiLoad 16/600 Superdex 75 prep grade column (GE Healthcare, Uppsala, Sweden); the final preparations were more than 95% pure based on the SDS-PAGE analysis.

OASS from *S. Typhimurium* (StOASS) was expressed in Rosetta™ 2 (DE3) cells with an N-terminal His$_6$-tag removable with TEV protease. Cells were lysed as described above in 50 mM sodium phosphate, 300 mM NaCl, pH 8.0, and the clarified supernatant was loaded on a Talon™ resin; the column was washed in the presence of 20 mM imidazole and the protein was eluted with 300 mM imidazole. Fractions containing purified StOASS were collected and put together in a 12 kDa dialysis tube in the presence of 1 mM EDTA, fivefold molar excess of PLP, and 1 mg of TEV protease every 50 mg of protein. After O/N dialysis, the solution was recovered, centrifuged to remove precipitated proteins, and loaded again on the Talon™ resin to separate cleaved tags, unreacted constructs and TEV protease. The final solution was concentrated and exchanged in 10 mM HEPES, 1 mM EDTA, 1 mM TCEP, pH 8.0.

OASS from *E. coli* (EcOASS) was expressed in Tuner™ (DE3) cells with a C-terminal His$_{10}$-tag. Cells were lysed as described above in 50 mM sodium phosphate, 300 mM NaCl, pH 7.0, and the clarified supernatant was loaded on a Talon™ resin; the column was washed in the presence of 20 mM imidazole and the protein was eluted with 300 mM imidazole. Fractions containing purified EcOASS were collected and put together in a 12 kDa dialysis tube in the presence of 1 mM EDTA and a fivefold molar excess of PLP. After O/N dialysis, the solution was recovered, centrifuged to remove precipitated proteins and finally exchanged and concentrated in 10 mM HEPES, 1 mM EDTA, pH 8.0.

### 3.3. UPAR415

The synthesis and the characterization of UPAR415 (MW 266.34 g/mol) has been carried out as described in [41]. The powder was dissolved in 100% dimethyl sulfoxide (DMSO) to a 100 mM stock solution and then serially diluted in 100 mM HEPES, pH 7.0, and tested at the reported concentrations, containing a maximum of 0.01% DMSO, except if otherwise stated.

### 3.4. SPR Measurements

SPR single-cycle kinetics (SCK) measurements were performed at 25 °C by using a Biacore X100 instrument (GE Healthcare, Uppsala, Sweden). CM5 sensor chips and reagents were purchased from GE Healthcare, except HEPES buffer, supplied by Sigma-Aldrich (S. Louis, MO, USA). Experiments were carried out in 100 mM HEPES, pH 7.0, in the presence of 0.005% P20 surfactant, as running buffer. For each experiment, flow cell 1 (Fc1) was prepared as flow cell 2 (Fc2), but without intermediate ligand immobilization, and used as a reference for refractive index changes and nonspecific interactions. Before each SCK, the ligand OASS was covalently immobilized by amine coupling onto the Fc2 chip surface with a manual run setup. Briefly, the dextran matrix of the sensor chip was initially equilibrated with running buffer by three priming cycles of five minutes and its carboxyl groups were activated by flowing a solution of 0.1 M N-hydroxysuccinimide (NHS) and 0.4 M N-ethyl-N-(3-dimethylaminopropyl) carboadiimide (EDC) at 10 µL/min for 420 s. A solution of 11.5 µg/mL OASS in 10 mM acetate buffer, pH 5, was then injected at 10 µL/min for the coupling reaction. A final amount of ligand equal to 140–350 RU (resonance units) was immobilized on each sensor chip. Finally, 1 M ethanolamine hydrochloride, pH 8.5, was injected at 10 µL/min for 420 s over both Fc1 and Fc2, to block the unreacted groups. SCKs were carried out by the sequential injection of five increasing concentrations of the SAT analyte (range 0.5–100 nM, monomer concentration), without any regeneration step between consecutive injections [67]. For each injection step, SAT samples were fluxed.
over the sensor chip surface for 160 s at 30 µL/min, followed by a 500 s dissociation step with running buffer; at the end of the cycle, a final dissociation step of 400 s in running buffer was performed. SPR competition experiments in the presence of OASS inhibitor were carried out as described above, but for each SCK, injection samples were prepared in the presence of a fixed concentration of UPAR-415 (range 0.15–2 µM). SCK sensorgrams were globally fitted by using the Langmuir 1:1 binding model [68,69], assuming a simple reversible bimolecular reaction and considering the mass transport effect that drives the analyte toward the chip surface (SAT<sub>surface</sub>) or back to the bulk solution (SAT<sub>bulk</sub>), with the same mass transfer coefficient k<sub>t</sub> [70]:

\[
\text{SAT}_{\text{bulk}} \xrightleftharpoons[\text{k}_i]{\text{k}_o} \text{SAT}_{\text{surface}} + \text{OASS} \quad \xrightleftharpoons[\text{k}_\text{off}]{\text{k}_\text{on}} \text{OASS} : \text{SAT}
\]

The binding of SAT to the ligand OASS-A brings to the formation of the CS complex, whose equilibrium depends on association (k<sub>on</sub>) and dissociation (k<sub>off</sub>) rate constants.

The temporal variation of analyte and ligand concentrations and of their complex was described by the following set of differential equations [71]:

\[
\frac{d[\text{SAT}_{\text{surface}}]}{dt} = k_t([\text{SAT}_{\text{bulk}}] - [\text{SAT}_{\text{surface}}]) - (k_{\text{on}}[\text{OASS}][\text{SAT}_{\text{surface}}] - k_{\text{off}}[\text{OASS}:\text{SAT}]) \quad (1)
\]

\[
\frac{d[\text{OASS}]}{dt} = -(k_{\text{on}}[\text{OASS}][\text{SAT}_{\text{surface}}] - k_{\text{off}}[\text{OASS}:\text{SAT}]) \quad (2)
\]

\[
\frac{d[\text{OASS}:\text{SAT}]}{dt} = (k_{\text{on}}[\text{OASS}][\text{SAT}_{\text{surface}}] - k_{\text{off}}[\text{OASS}:\text{SAT}]) \quad (3)
\]

The goodness of the fits was evaluated by χ² values and residual plots.

3.5. Fluorescence Measurements

Fluorescence binding experiments were performed on a Fluoromax-4 (Horiba Jobin-Yvon, Kyoto, Japan), or an FS5 (Edinburgh Instruments Ltd., Livingston, UK) spectrofluorometers equipped with a thermostated water bath. Measurements were carried out at 25 °C in a 3 mm pathlength quartz microcuvette in 100 mM HEPES buffer, pH 7.0. Before every experiment, proteins were spectrophotometrically quantified on a Cary 4000 spectrophotometer (Agilent, Santa Clara, CA, USA) by their extinction coefficients at 280 nm (SAT, S. Typhimurium 26930 M<sup>−1</sup> cm<sup>−1</sup>; E. coli 26930 M<sup>−1</sup> cm<sup>−1</sup>), determined by ProtParam software [72] or 412 nm (OASS-A), corresponding to the internal aldimine maximum emission wavelength (S. Typhimurium 9040 M<sup>−1</sup> cm<sup>−1</sup>; E. coli 9375 M<sup>−1</sup> cm<sup>−1</sup> [31]). OASS-A solutions (100 nM) were titrated with increasing amounts of SAT in the presence of a fixed concentration of UPAR415 inhibitor (0.5, 5, or 10 µM). After each addition of SAT, the solution was incubated for 2 min to equilibrate the complex formation. The blue shift related to UPAR415 displacement from OASS active site by SAT was followed by collecting emission spectra upon PLP excitation at 412 nm.

Emission wavelength maxima were fitted to a binding isotherm to determine the apparent dissociation constant <sup>D</sup>app of CS complex [73]. The intrinsic dissociation constant is obtained by the equation:

\[
K_D = \frac{K_{D,\text{app}}}{1 + \frac{[L]}{K_{D,L}}}
\]

where L is represented by UPAR415 and K<sub>D,L</sub> is its dissociation constant at 25 °C.

The dissociation constants of UPAR415 for OASS-A were determined in 100 mM HEPES buffer, pH 7.0, in the presence of 5% DMSO. Emission spectra upon excitation at 412 nm of solutions containing 365 nM StOASS-A or 385 nM EcOASS-A in the presence of increasing concentrations of UPAR415 were collected, and fluorescence intensities at 500 nm were plotted to inhibitor concentration. Data were fitted to a quadratic equation describing tight binding:
\[ I = I_0 + a \times \frac{[\text{OASS} - A] + [\text{U415}] + K_D - \sqrt{([\text{OASS} - A] + [\text{U415}] + K_D)^2 - (4 \times [\text{OASS} - A] \times [\text{U415}])(5\right)

where \( I \) is the fluorescence intensity at 500 nm, \( I_0 \) is a horizontal offset, \( a \) is the maximum change in fluorescence at saturating ligand, and \( K_D \) is the dissociation constant of OASS-A:UPAR415 complex.

4. Conclusions

The rapid spreading of bacterial resistances urges the search of novel antimicrobial compounds and antibiotic adjuvants. In this context, the metabolism of cysteine represents an attractive and promising target to undermine bacterial abilities of adaptation. Indeed, the reducing properties of cysteine are employed as an effective response to the oxidative stress experimented by bacteria during persistence phases in the host and induced by antibiotics. Several studies have illustrated the upregulation of different genes of cysteine metabolism and, on the other hand, an increased susceptibility to antimicrobial agents of strains characterized by an impaired oxidative stress response [3,7,8,74]. In this framework, in the last years, cysteine biosynthetic pathway has been the object of several drug discovery campaigns. A particular attention has been addressed to SAT and OASS-A, the two enzymes catalyzing the last two steps in cysteine biosynthesis in bacteria. SAT and OASS-A can assemble in a finely regulated bi-enzymatic complex known as cysteine synthase (CS), whose formation and regulation affect the single enzyme activities and finely tune many aspects of bacterial fitness and responses to external stimuli, comprising defense and protection mechanisms. In this context, a recent drug discovery campaign individuated the molecule UPAR415 as the most potent synthetic inhibitor for OASS-A developed so far. Recently, we demonstrated the viability of targeting OASS-A, showing through microbiological studies that the inhibition of this enzyme has synergic or additive effects with the last-line antibiotic colistin. Besides these results and the previous biochemical studies in vitro on OASS-A [41], the broader impact of UPAR415 on cysteine metabolism has to be further investigated. In this work, we exploited surface plasmon resonance and static fluorescence measurements to analyze the influence of UPAR415 on the complex formation between SAT and OASS-A from \( S. \) Typhimurium and \( E. \) coli. Experimental results demonstrated the ability of the inhibitor, initially developed for \( S. \) Typhimurium OASS-A inhibition, to decrease the apparent affinity of the CS complex of both bacteria by interfering with SAT binding to OASS-A active site. This effect could impact the action of SAT in vivo, whose activity is maximized when bound to OASS-A, thus limiting the supply of OAS and altering the regulation of cysteine regulon. Indeed, it has been reported that mutations of SAT that affect CS complex formation exert pleiotropic effects on OASS-A activity, resulting in cysteine auxotrophy [28]. This study paves the way for a deeper understanding of the effect of OASS inhibitors on the activity and regulation of cysteine biosynthetic pathway in vivo. In perspective, an updated knowledge of the inhibition mechanisms of OASS and SAT might guide the design of more potent compounds, susceptible to impact not only the synthesis of cysteine but also the full range of regulatory activities related to CS complex.

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