Disulfide Bond Formation and Activation of *Escherichia coli* β -Galactosidase under Oxidizing Conditions

Joaquín Seras-Franzoso,^{a,b,c} Roman Affentranger,^{a*} Mario Ferrer-Navarro,^a Xavier Daura,^{a,d} Antonio Villaverde,^{a,b,c} and Elena García-Fruitós^{a,c}

Institut de Biotecnologia i de Biomedicina, Universitat Autònoma de Barcelona, Bellaterra (Cerdanyola del Vallès), Barcelona, Spain^a; Departament de Genètica i de Microbiologia, Universitat Autònoma de Barcelona, Bellaterra (Cerdanyola del Vallès), Barcelona, Spain^b; CIBER de Bioingeniería, Biomateriales y Nanomedicina (CIBER-BBN)^c; and Institució Catalana de Recerca i Estudis Avançats (ICREA), Barcelona, Spain^d

Escherichia coli β -galactosidase is probably the most widely used reporter enzyme in molecular biology, cell biology, and biotechnology because of the easy detection of its activity. Its large size and tetrameric structure make this bacterial protein an interesting model for crystallographic studies and atomic mapping. In the present study, we investigate a version of *Escherichia coli* β -galactosidase produced under oxidizing conditions, in the cytoplasm of an Origami strain. Our data prove the activation of this microbial enzyme under oxidizing conditions and clearly show the occurrence of a disulfide bond in the β -galactosidase structure. Additionally, the formation of this disulfide bond is supported by the analysis of a homology model of the protein that indicates that two cysteines located in the vicinity of the catalytic center are sufficiently close for disulfide bond formation.

Since the inception of recombinant DNA technology, a large number of proteins of economic and therapeutic interest have been produced using *Escherichia coli* as a cell factory. The conformational quality of the proteins expressed is controlled by a complex network of chaperones and proteases, and in fact, this parameter determines the potential biotechnological use of the product obtained (31, 35, 50, 52).

Nowadays, the wide spectrum of cloning and gene expression vectors and mutant strains, and the knowledge of bacterial metabolism, allows the expression of an enormous range of proteins, whether prokaryotic or eukaryotic. Specifically, genetically modified *E. coli* strains have contributed significantly to the expression of soluble recombinant proteins, proving that the genetic background is extremely important for recombinant protein expression (29, 49, 50, 53). In this context, since prokaryotes have a reducing cytoplasm and since proteins that require disulfide bond formation to reach their functional conformation cannot be active under these conditions (9), many strategies have been developed for the production in *E. coli* of proteins containing disulfide bonds in their stable native structure (7). It has been reported that a considerable number of factors are involved in the thiol-disulfide balance in the *E. coli* cytoplasm (37); the thioredoxin system (which consists of thioredoxin reductase [encoded by *trxB*] and thioredoxin [*trxA*]) and the glutaredoxin system (which consists of glutaredoxin reductase [*gor*], glutathione [*gshA*, *gshB*], and three glutaredoxins [*grxA*, *grxB*, *grxC*]) are the main pathways (32, 37). One of the most common approaches that allow the folding of proteins requiring the formation of disulfide bridges is the use of the Origami strain. Origami (*gor trxB* mutant) is a double mutant strain in which the thioredoxin/glutaredoxin reductase pathway is knocked out, resulting in an oxidizing cytoplasm, compatible with the folding of proteins whose structure and function depend on the correct formation of cysteine-cysteine bridges (7). Besides the use of the Origami strain as a host cell, other strategies are also used to promote the production of proteins with disulfide bonds in bacteria. Since the periplasm is an oxidizing compartment, the export of proteins from the cytoplasm to the periplasm is a widely

used alternative (23, 31). In many cases, however, this strategy results in a lower protein yield than that obtained by expression in the cytoplasm. It has also been shown that, in addition to producing the protein in an Origami strain or in the periplasm to allow the formation of disulfide bonds (27), the fusion of solubilizing carriers (e.g., Sumo) to the desired protein can improve the quality of the expressed protein (53). Another frequently used method is the coexpression of chaperones/foldases during the production process (44).

Disulfide bond formation is, in many cases, essential for the production of functional proteins (5, 9, 38, 47) and, in other situations, for the improvement of folding and therefore for enhancement of the activity of the desired protein (1). Although disulfide bonds are rarely formed in bacterial cytoplasmic proteins, there are some surprising exceptions to this general rule (1, 18, 28, 51). In this context, some studies performed by our group (unpublished data) and data published by others (39, 46) seem to suggest that thiols inactivate *E. coli* β -galactosidase. It is noteworthy that all the known structures of β -galactosidase have been solved in the presence of the reducing agent dithiothreitol (DTT) (19–21), and the activity and structure of β -galactosidase have never been explored under oxidizing conditions. *E. coli* β -galactosidase (EC 3.2.1.23) forms a tetramer and has been one of the most widely used reporter enzymes for many applications, such as analysis of the regulation of gene expression, characterization of protein function/structure, and analysis of target gene expression (6, 48). On the other hand, bacterial β -galac-

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Address correspondence to Elena García-Fruitós, efruitos@ciber-bbn.es.

* Present address: R&D, Douglas Connect, Zeiningen, Switzerland.

† For this virtual institution, see <http://www.ciber-bbn.es>.

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tosidases also have enormous potential in the food industry (14, 16). Moreover, β -galactosidase can hydrolyze not only lactose, resulting in glucose and galactose, but also other substrates, yielding colored products, thus making the enzyme a suitable reporter for the applications mentioned. Considering the enormous potential of β -galactosidase and the possible effects of redox conditions on protein functionality, we decided to investigate whether a suitable genetic background might improve its folding. In this study, we measured the activities of a recombinant *E. coli* β -galactosidase (r β -galactosidase) in two *E. coli* strains: a wild-type and an Origami strain. A recombinant green fluorescent protein (rGFP), which does not form any disulfide bond, was used as a control in all the experiments performed. Our data show that the specific enzymatic activity of β -galactosidase is unexpectedly improved under oxidizing conditions and strongly support the idea that cysteines 500 and 536 of *E. coli* β -galactosidase form a disulfide bond *in vivo* when produced in the Origami strain.

MATERIALS AND METHODS

Strains and plasmids. The *Escherichia coli* strains used in this work were the K-12 derivatives MC4100 [*araD139* Δ (*argF-lac*)*U169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR Str^r*] (43), Origami 2 [Δ (*ara-leu*)7697 Δ *lacX74* Δ *phoA* *PvuII phoR araD139 ahpC galE galK rpsL F'*[*lac⁺ lacI^q pro*] (DE3) *gor522::Tn10 trxB Str^r Tet^r*] (bioNova Científica s.l.), and XL10-Gold [*Tet^r Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac Hte F'*[*proAB lacI^qΔM15 Tn10(Tet^r) Kan^r*]] (Stratagene).

The plasmids used were pTVP1LAC (Ap^r) and pTVP1GFP (Ap^r) (10), encoding r β -galactosidase and rGFP, respectively, fused to the foot-and-mouth disease virus VP1 capsid protein. The expression of recombinant genes in both plasmids is under the control of an isopropyl- β -D-thiogalactoside (IPTG)-inducible *trc* promoter.

Culture conditions, cell disruption, and cell fractionation. Protein was produced in shake flask bacterial cultures growing at 37°C and 250 rpm in rich LB medium (43) with the required antibiotics. Once the absorbance at 550 nm reached 0.5, gene expression was induced by addition of 1 mM IPTG. Cell samples were collected 2 h later for further analysis. Data were obtained from three or more independent experiments and were submitted to a *t* test analysis.

Protein fractionation. Cells from 2-ml culture samples were collected by low-speed centrifugation at 4°C (15 min at 15,000 $\times$ g) and were resuspended in 500 μ l phosphate-buffered saline (PBS) buffer with protease inhibitor cocktail (reference no. 1,836,170; Roche). These samples were disrupted with a MagNA Lyser instrument (Roche) for 20 s at 3,000 rpm. After centrifugation at 4°C for 15 min at 15,000 $\times$ g, the supernatants were collected and were used directly for analysis of the soluble protein.

Determination of fluorescence and specific fluorescence. Fluorescence was measured in a Cary Eclipse fluorescence spectrophotometer (Varian) at 510 nm by using an excitation wavelength of 450 nm. Fluorescence measurements were carried out in triplicate and were corrected by either biomass or amounts of rGFP (determined as described below).

Determination of enzymatic activity and specific enzymatic activity. β -Galactosidase activity was determined in PBS through a variant of Miller's protocol (33) by monitoring the colorimetric signal produced by *ortho*-nitrophenyl- β -galactoside (ONPG) degradation at 420 nm. To determine the presence of disulfide bonds, a β -galactosidase enzymatic assay was performed with increasing concentrations of DTT (10 mM and 20 mM) and in the absence of reducing agents. The results presented are averages for at least three independent experiments. Activity values are corrected by either biomass or amounts of r β -galactosidase protein (determined as described below).

Quantitative protein analysis. Samples were diluted in denaturing buffer (26) at appropriate ratios. After the soluble fraction was boiled for 15 min and the total fraction for 30 min, samples were loaded onto dena-

turing gels for Western blot determination, using as primary antibodies a polyclonal serum against GFP (Santa Cruz Biotechnology) or a polyclonal serum against β -galactosidase (MP Biomedicals, Inc.), depending on the protein to be analyzed. The secondary antibody was always an anti-rabbit antibody (Bio-Rad). Once the blots were dried, they were scanned at high resolution, and bands were quantified with Quantity One software (Bio-Rad), using known concentrations of commercial GFP or β -galactosidase as controls. Protein concentrations were used to calculate specific activity or fluorescence.

Native gels. To determine disulfide bond formation, r β -galactosidase (135-kDa) samples in either 0.25 M Tris-HCl–87% glycerol–6 mg bromophenol blue and distilled water or a 20 mM DTT buffer (25) were incubated at 37°C for 15 min and were loaded onto a nondenaturing 6% polyacrylamide gel for Western blot determination as described above. Three replicas were loaded onto each gel (25), and samples A and B were run in the same gel under the same exact conditions. As a reference (C+), we used a commercial *E. coli* β -galactosidase with a molecular mass of 116 kDa.

Modeling. Homology models of the β -galactosidase used in this work were constructed using MODELLER 9v2 software (42). As a template structure, we used the coordinates of the *E. coli* β -galactosidase with entry 1JYV (19) in the Brookhaven Protein Data Bank (PDB). In what follows, we adhere to the numbering of residues corresponding to the sequence deposited for 1JYV. Alignment of the 1,011 residues (R13 to K1023) covered by 1JYV to the sequence of the protein used in this work results in 983 (97.2%) identical residues. We computed 50 different homology models of the reduced β -galactosidase using a very thorough VTFFM (variable target function method with conjugate gradient) schedule and very thorough molecular dynamics refinement (molecular dynamics with simulated annealing). The following settings of MODELLER's automodel class were used: library schedule, autosched. slow; max_var_iterations, 300; md_level, refine_very.slow; repeat_optimization, 5; max_molpdf, 1e6. The resulting models were ranked using the discrete optimized protein energy (DOPE) potential (45).

The part of the β -galactosidase sequence covered by the alignment with 1JYV contains 15 cysteine residues. Generating the distance matrix of all pairs of cysteine sulfur atoms, we identified two pairs separated by a distance of <1 nm: C500–C536 (0.52 nm) and C389–C402 (0.68 nm). To evaluate the structural changes involved in, and possibly necessary for, disulfide bond formation, we generated 50 homology models incorporating either the disulfide bond C500–C536 alone or both C500–C536 and C389–C402 simultaneously. In both cases, the same settings were used as in the calculation of the homology models of the reduced form of the protein. Again, the resulting models were scored using the DOPE potential.

β -Galactosidase mutagenesis. A single mutation in C500 of r β -galactosidase was carried out by inverse PCR producing a single nucleotide replacement (Cys to Ala). The PCR primers used were CCACCGATATT ATTGCCCGGATGTACGCGC (forward) and GCGCGTACATCGGGG CAATAATATCGGTGG (reverse) (underlining indicates nucleotides that change Cys to Ala).

The PCR product was then treated with DpnI for 2 h at 37°C to remove parental DNA and was transformed into *E. coli* XL10-Gold cells. Positive clones were selected by sequencing and were transformed to the *E. coli* MC4100 and Origami strains for further analysis.

Chemical modification of β -galactosidase. β -Galactosidase was reduced by adding 2 μ l of a 44 mM DTT solution to 2 μ l of a solution containing 4 μ g of β -galactosidase. Reduction was performed for 1 h at 37°C. β -Galactosidase was oxidized using 4 mM H₂O₂ at room temperature as follows: 2 μ l of 8 mM H₂O₂ (in 100 mM NH₄HCO₃) was added to a 2- μ l solution containing 4 μ g of β -galactosidase. For β -galactosidase carboxamidomethylation, three different samples were treated with iodoacetamide (IAA): β -galactosidase in Milli-Q water, β -galactosidase treated with DTT, and β -galactosidase treated with H₂O₂. Two microli-

FIG 1 Specific biological activity (measured by either absorbance or fluorescence). The specific activities of r β -galactosidase (A) and rGFP (B) produced in both the wild-type (wt [MC4100]) and Origami *E. coli* strains were determined. The absolute values for biological activity are given above the bars. The *P* values, obtained by a *t* test, are 0.184 (A) and 0.060 (B).

ters of each sample was incubated with iodoacetamide at a final concentration of 100 mM in 50 mM NH_4HCO_3 .

Proteolytic degradations of β -galactosidase and its carboxamidomethylated derivatives were carried out in 50 mM NH_4HCO_3 , pH 8. Trypsin (Promega, Madison, WI) was added to a final enzyme-to-substrate ratio of 1:50. Digestions were performed for 3 h at 37°C. The reactions were terminated by adding trifluoroacetic acid (TFA) to a final concentration of 0.2%.

MS analysis. For matrix-assisted laser desorption ionization (MALDI) analysis, 1 μl of sample was mixed with the same volume of a saturated solution of α -cyano-4-hydroxy-*trans*-cinnamic acid matrix (0.3 mg/ml in H_2O -acetonitrile-TFA at 6:3:1), and the mixture was spotted onto a MALDI target plate (Bruker Daltonics, Bremen, Germany). The drop was air dried at room temperature. MALDI mass spectra were recorded in the positive-ion mode on an Ultraflex Extreme time-of-flight instrument (Bruker Daltonics, Bremen, Germany). Ion acceleration was set to 25 kV. All mass spectra were externally calibrated using a standard peptide mixture containing angiotensin II (1,046.54), angiotensin I (1,296.68), substance P (1,347.74), bombesin (1,619.82), rennin substrate (1,758.93), adrenocorticotrophic hormone fragment residues 1–17 (2,093.09), adrenocorticotrophic hormone fragment residues 18–39 (2,465.20), and somatostatin 28 (3,147.47). Calibration was considered good when a value below 1 ppm was obtained. The spectra were processed using Flex Analysis software, version 2.2 (Bruker Daltonics). The SNAP algorithm included in the software was used to select the monoisotopic peaks from the isotopic distributions observed. For peptide mass fingerprinting (PMF) analysis, the MASCOT search engine (Matrix Science, London, United Kingdom) was used with the following parameters: one missed cleavage permission, 50-ppm measurement tolerance, and at least four matching peptide masses. Positive identifications were accepted with *P* values lower than 0.05. In the searches, methionine residues modified to methionine sulfoxide were allowed, and cysteine residues were allowed to be reduced and alkylated by iodoacetamide to carboxyamidomethyl cysteine wherever necessary. For tandem mass spectrometry (MS-MS) analysis, the same equipment was used. For database searches, the MASCOT search engine was also used with 50 ppm of error in MS and 0.3 Da in MS-MS. Modification of methionine sulfoxide and carboxyamidomethyl cysteine was also allowed in the searches.

RESULTS

Although both Reithel and coworkers and Shifrin and collaborators reported that β -galactosidase is rapidly inactivated in the presence of thiols (39, 46), the possible presence of a disulfide bond in the enzyme structure had never been explored in detail before.

Therefore, in this study, we have analyzed the effect of the oxidizing/reducing background on the structure and functionality of *E. coli* β -galactosidase by measuring the specific activity of the enzyme under both conditions, when expressed in an Origami strain and in a wild-type strain (Fig. 1A). Interestingly, r β -galactosidase exhibited higher specific activity in the Origami strain than in the wild-type strain (Fig. 1A), showing that the mutations in thioredoxin reductase and glutaredoxin reductase clearly have a positive effect on its activity. On the other hand, when determining the specific fluorescence of rGFP, used as a control, we observed that the protein produced in the *E. coli* wild-type strain showed a higher specific fluorescence than that produced in the Origami strain (Fig. 1B). This observation indicates that the increase in the specific activity of protein is not a general event that takes place in the Origami cytoplasm but a specific fact related to the redox environment.

Analyzing the amount of soluble protein produced under each condition, we observed that larger amounts of both rGFP (Fig. 2B) and r β -galactosidase (Fig. 2A) are produced in the Origami strain than in the wild-type strain (Fig. 2B). Therefore, the expression of this bacterial β -galactosidase in an oxidizing environment resulted not only in higher specific activity (Fig. 1A) but also in a higher soluble protein yield (Fig. 2A). Additionally, although the soluble protein yield improves when the Origami strain is used, the total-protein yield obtained is lower ($7.28 \pm 1.28 \text{ ng}/\mu\text{l}$) than that in the wild-type strain ($21.77 \pm 4.17 \text{ } \mu\text{g}/\mu\text{l}$), indicating that the degree of aggregation of r β -galactosidase is much higher under reducing conditions. Thus, we can conclude that by using oxidizing conditions, it is also possible to enhance solubility, avoiding, at least partially, the formation of protein aggregates known as inclusion bodies (10).

Since the data presented here indicate that an oxidizing background favors *E. coli* β -galactosidase activity and productivity and that this could be related to the formation of a disulfide bond, we decided to study the possible presence of one or more cysteine-cysteine bridges in r β -galactosidase when expressed in the Origami strain. For that purpose, native polyacrylamide gels under reducing and nonreducing conditions were analyzed in order to determine, by an alternative method, the eventual formation of disulfide bonds in r β -galactosidase. Proteins containing cysteine-cysteine bridges (oxidized form) migrate faster on a nondenatur-

FIG 2 Amounts of soluble protein observed by Western blotting. r β -Galactosidase (A) and rGFP (B) were produced in the wild-type (wt [MC4100]) and Origami strains. The *P* values, obtained by a *t* test, are 0.056 (A) and 0.076 (B).

ing gel than proteins in the reduced form (8, 9, 36), due to decreases in hydrodynamic volume and flexibility (24). Then, as presumed (8, 36), and considering that protein migration on native gels depends not only on the molecular weight but also on the conformation of the protein, in the absence of a reducing agent, the mobility of the reduced protein was clearly lower than that of the protein produced in the Origami strain (Fig. 3A). Thus, the results obtained prove that the redox environment during protein expression can modify the protein conformation, presumably by the formation of the target disulfide bond.

Interestingly, the differences observed in protein mobility between the two strains strongly decreased in the presence of DTT (Fig. 3B). Therefore, since the difference observed in protein migration is due to a modification of production parameters, but not of the protein amino acid sequence, all these data fit with the possible presence of a disulfide bond in the β -galactosidase structure.

To additionally confirm better protein folding in Origami cells, we monitored the enzymatic activity of the enzyme with and without DTT. We evaluated the enzymatic activity of r β -galactosidase at different DTT concentrations, ranging from 0 to 20 mM. In Fig. 4 we show that, as previously reported, in the absence of DTT, *E. coli* β -galactosidase produced in the Origami strain exhibited higher activity than that produced in the wild-type strain. Moreover, our results also show that r β -galactosidase activity was significantly reduced in the presence of increasing concentrations of

DTT and that this reduction was more progressive in the Origami than in the wild-type strain (Fig. 4). Catalytic activity decreased in both cases in the presence of increasing concentrations of DTT, reaching the same specific activity value at 20 mM DTT (Fig. 4). Although this reduction was more marked in the Origami strain, wild-type β -galactosidase is also affected by the presence of this reducing agent. This could indicate that even in a reducing environment, such as *E. coli* cytoplasm, a fraction of the enzyme produced forms disulfide bonds, as has been reported for other proteins (1, 18, 28, 51) and as might also occur with the natural β -galactosidase protein.

To further investigate the possible formation of disulfide bonds in *E. coli* β -galactosidase, we modeled this protein under oxidizing and reducing conditions. As expected from the very large number of identical residues between the r β -galactosidase used in this work and the sequence covered by PDB entry 1JYV (19), the homology models computed for the reduced form and 1JYV are structurally nearly identical (Fig. 5A). Interestingly, incorporation of a disulfide bond between cysteines 500 and 536 does not require large conformational changes. In fact, both in the model of the reduced form and in 1JYV, with a simple rotation

FIG 3 Nondenaturing polyacrylamide electrophoresis gel of r β -galactosidase produced in the wild-type (wt [MC4100]) and Origami strains. Gels were analyzed in the absence (A) or presence (B) of the reducing agent DTT. R1, R2, and R3 correspond to three Origami replicas, while R4, R5, and R6 correspond to three replicas from the wild-type strain. C+ corresponds to a commercial *E. coli* β -galactosidase. Arrows indicate the positions of the electrophoretic bands.

FIG 4 Specific enzymatic activities of r β -galactosidase produced in the wild-type (wt [MC4100]) and Origami strains. Specific activity was determined without DTT (filled bars) and with increasing concentrations of DTT: 10 mM (light shaded bars) and 20 mM (dark shaded bars). The *P* values, obtained by a *t* test, are 0.028 for the wt strain and 0.043 for the Origami strain.

FIG 5 β -Galactosidase modeling under oxidizing conditions. (A) (Top) Illustration of the conformational changes involved in the formation of a disulfide bond between cysteines C500 and C536. Represented are the residues with a minimum distance of <0.5 nm from the two cysteines. The carbon atoms of C500 and C536 are shown in green, and those of all other residues are displayed in white. Oxygen, nitrogen, and sulfur atoms are colored red, blue, and yellow, respectively. Thick sticks represent the best model (according to its DOPE potential) with the disulfide bond formed; thin sticks represent the best model of the reduced form. The template structure (PDB entry 1JYV) is drawn with black lines. (Bottom) Histograms of the DOPE scores of the 50 models obtained for reduced β -galactosidase and of the 50 models obtained with the disulfide bond between C500 and C536. (B) Illustration of the close proximity between the C500-C536 disulfide bond and the active-site residues (E461, Y503, E537, H540, G794). The β -galactosidase backbone is drawn as a white cartoon representation. The side chains of the two cysteine residues involved in the disulfide bond (green) and of the active-site residues (light red) are shown as solid sticks surrounded by semitransparent spheres. Oxygen, nitrogen, and sulfur atoms are colored red, blue, and yellow, respectively.

around the side chain ($C-C_{\alpha}-C_{\beta}-S$) dihedral angle of the cysteine residues in question, the two sulfur atoms can easily be brought within bonding distance. The atomic packing in the surrounding region is even low enough to allow such a position without atomic overlaps. Therefore, not surprisingly, the quality of the models (according to the DOPE potential) with a disulfide bond connecting residues 500 and 536 cannot be distinguished from that of the models of the reduced form of the protein (Fig. 5A).

The situation is different for the second disulfide bond whose formation was explored (C389-C402). Although the two sulfur atoms are only slightly farther apart in the reduced models than those for the C500-C536 pair (0.68 nm in the former; 0.52 nm in the latter), disulfide bond formation is more difficult, because the two residues are already oriented toward each other. Thus, it can be anticipated that the disulfide bond between C389 and C402 cannot be formed without distorting the backbone conformation at least locally. Indeed, defining a standard disulfide bond (with a bond length of 0.2 nm) between the two sulfur atoms for the modeling procedure was not sufficient to bring them closer than 0.24 nm (results not shown). While this does not exclude the

possibility of disulfide bond formation between C389 and C402, it indicates that a disulfide bond would not be formed as easily as between C500 and C536.

These modeling calculations strongly suggest that at least the disulfide bond between cysteines 500 and 536 could be formed in a nonreducing environment. That the presence of this disulfide bond might affect the activity of β -galactosidase is indicated by its proximity to the catalytic center of the enzyme (Fig. 5B). C536 is adjacent to glutamate 537, and C500 is very close in sequence to tyrosine 503. The rigidity of this part of the active site brought about by the formation of the disulfide bond might increase the activity of the protein, in accordance with the experimental data obtained (Fig. 1A). Indeed, rigidity appears to be an evolutionarily favored characteristic of enzymatic active sites, since active sites have been shown to be significantly more rigid than other protein regions (3, 55). In this case, E537 acts as the nucleophile of the hydrolysis reaction catalyzed by β -galactosidase (11, 54), while Y503 plays the role of an acid catalyst for the cleavage of the covalent bond between E537 and galactose (34, 40, 41). These two catalytic residues act cooperatively on the same step of the hydro-

FIG 6 Specific activities of r β -galactosidase (filled bars) and r β -galactosidase C500A (open bars). r β -Galactosidase activities (expressed as the percentage of β -galactosidase activity in r β -galactosidase C500A with respect to the activity of wild-type r β -galactosidase) were determined in both the wild-type (wt [MC4100]) and Origami *E. coli* strains. The *P* values, obtained by a *t* test, are 0.086 for the wt and 0.040 for the Origami strain.

lysis reaction (41), and rigidification of their relative positions by a disulfide bond could explain the increased activity of the protein when expressed under oxidizing conditions.

Since the results obtained seem to indicate that cysteines 500 and 536 play a key role in the formation of a disulfide bond, and since it is widely accepted that the mutation of one of the two cysteines is sufficient to determine the formation of a disulfide bond (13), C500 was changed to Ala. The results show that the specific activity values observed for the mutated r β -galactosidase are lower than those observed for the wild-type enzyme (Fig. 6). Interestingly, we observed that this decrease is particularly marked in the Origami strain (around 85%). Thus, all these data, in agreement with all the previous results, strongly support the involvement of C500 and C536 in the formation of a disulfide bond. It should be noted that involvement of this cysteine in a disulfide bond other than that proposed is unlikely without a large rearrangement of the monomeric protein structure.

Furthermore, in order to finally confirm the presence of a disulfide bond between C500 and C536, a method based on mass spectrometry was used (2). To obtain the peptide maps, β -galactosidase in Milli-Q water, DTT-reduced β -galactosidase, and H₂O₂-oxidized β -galactosidase were carboxamidomethylated and were subsequently cleaved proteolytically with trypsin. The resulting peptide mixtures were analyzed by MALDI-MS peptide mapping. Trypsin cleavage yielded a pattern of 55 different peptides. Both cysteine residues 500 and 536 were found completely alkylated in the reduced sample as well as in β -galactosidase in Milli-Q water (Fig. 7). This indicated that these two cysteine residues are not involved in the formation of a disulfide bridge under these conditions. In order to assign these ion signals unequivocally, their MS-MS spectra were obtained (Fig. 7). MALDI-MS peptide mapping after tryptic digestion of the oxidized β -galactosidase and the oxidized and alkylated β -galactosidase showed the presence of almost all individual peptides except for the two ion signals containing residues C500 (*m/z*, 3,127.51 Da) and C536 (*m/z*, 3,188.671 Da) (Fig. 8A). The absence of these signals indicates that these two cysteines are linked with a disulfide bridge under these conditions. Furthermore, an ion sig-

nal of 6,234.546 Da was detected (Fig. 8B) and was assigned as a disulfide-bonded dipeptide containing the peptides 447-SVDPSR PVQYEGGGADTTATDIICPMYAR-505 and 523-WLSLPGETR PLILCEYAHAMGNSLGGFAK-551, where the two methionine residues have undergone oxidation.

DISCUSSION

Since it is widely accepted that the formation of cysteine-cysteine bridges is favored under oxidizing conditions, disulfide bonds of recombinant proteins usually cannot be formed in the reducing cytoplasm of bacteria. Thus, *E. coli* mutant strains lacking the main components of the redox pathway are widely used in order to produce heterologous proteins that require a proper oxidation status of cysteine residues to attain their native structures. In fact, the oxidation of cysteines in the *E. coli* cytoplasm is highly disfavored, due not only to the low redox potential but also to the absence of enzymes that catalyze protein thiol oxidation in the cytoplasm (4). In this context, it is generally accepted that the double mutant TrxB[−] Gor[−] strain (Origami), which has an intracellular environment shifted toward a more oxidative state, is the most useful cell factory for the production of proteins that contain disulfide bonds (22) and a good tool for understanding the role of disulfide bond formation in recombinant proteins.

On the other hand, β -galactosidase is a tetrameric bacterial protein of four identical subunits essentially composed of α -helices (17) that has been used as a reporter for many years. In this study, we report for the first time that the *E. coli* β -galactosidase enzyme forms a disulfide bond when produced in an oxidizing background, such as the cytoplasmic environment provided by the *E. coli* Origami strain. In this context, our model suggests not only that a disulfide bond is formed but also that it is located close to the catalytic center. Our results prove that β -galactosidase produced in the Origami strain shows the highest specific activity, suggesting that the absence of thioredoxin reductase and glutaredoxin reductase clearly improves the microbial enzyme activity. In a previous study, Derman and Beckwith (8) used a TrxB[−] mutant strain to produce β -galactosidase, observing a decrease in the enzymatic activity of this strain compared to that of the control. This behavior could be explained by considering that it is not clear that proteins containing disulfide bonds are successfully produced in a TrxB[−] strain. The cytoplasm of this strain is at least partially reducing due to the presence of the still-functional glutathione reductase (15), which has overlapping functions with thioredoxin reductase in preventing disulfide bond formation (1). Therefore, our data also confirm, as reported in the literature, that the formation of disulfide bonds is more favorable in the cytoplasm of a TrxB[−] Gor[−] mutant strain than in that of a TrxB[−] mutant strain (4).

Although solubility has been taken universally as an indicator of protein conformational quality, it has been proposed recently that specific activity, and not solubility, should be used as a marker of protein quality (12). It has also been shown recently that conditions that enhance protein quality often reduce the final amounts of protein produced (12, 30). The comparative analysis performed in this study, surprisingly, shows that conformational quality and solubility are improved simultaneously when *E. coli* r β -galactosidase is expressed in the Origami strain. In contrast to that observation, it has been reported that both parameters cannot be improved simultaneously for proteins that do not need post-translational modifications (30), such as disulfide bond forma-

FIG 7 (Top spectrum) Treatment of β -galactosidase with iodoacetamide. Peaks containing C500 (m/z , 3,127.489) and C536 (m/z , 3,188.627 and 3,316.738) are indicated. (Central and bottom spectra) MS-MS for the 3,127.489-Da (central) and 3,188.627-Da (bottom) peaks.

FIG 8 (A) Details of the spectra obtained for different β -galactosidase treatments. (Top spectrum) β -Galactosidase treated with DTT and IAA. The carboxamidomethylated peptides containing C500 and C536 can be observed. (Central spectrum) β -Galactosidase treated with H_2O_2 . Under oxidizing conditions, the peaks containing C500 and C536 are not observed. (Bottom spectrum) β -Galactosidase treated with H_2O_2 and IAA. Under oxidizing conditions, alkylation of C500 and C536 does not take place. (B) Details of spectra obtained for different β -galactosidase treatments. After treatment with DTT alone or DTT plus IAA (top and central spectra, respectively), the peptide containing the disulfide bridge formed by C500 and C536 is not detected, but it appears in the H_2O_2 -treated sample (bottom spectrum).

tion. Thus, our results show that in those proteins that can reach a more stable conformation under specific conditions, such as an oxidizing background, both properties can be improved simultaneously.

To sum up, in this study we demonstrate, for the first time, an enhancement of β -galactosidase activity when the enzyme is produced in an oxidizing environment and, consequently, the formation of a disulfide bond in *E. coli* β -galactosidase under these con-

ditions. Our experimental data—supported and rationalized by modeling calculations—show not only that the oxidative environment in the *E. coli* cytoplasm provided by the Origami strain promotes disulfide bond formation in r β -galactosidase but also that this phenomenon tends to enhance the biological activity of r β -galactosidase. Moreover, our data also suggest that, although the formation of disulfide bonds is not favored under reducing conditions, a small percentage of the natural β -galactosidase produced in a reducing *E. coli* cytoplasm could form a C500-C536 disulfide bond, as occurs with other proteins (1, 18, 28, 51). This event would be compatible with the protein function, since we have observed that, although β -galactosidase shows higher specific activity under oxidizing conditions, it can also carry out its function under natural reducing conditions.

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