J Biol Inorg Chem (2004) 9: 396–402
DOI 10.1007/s00775-004-0536-1

ORIGINAL ARTICLE

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Interaction kinetics of the copper-responsive CopY repressor with the *cop* promoter of *Enterococcus hirae*

Received: 14 January 2004 / Accepted: 26 February 2004 / Published online: 1 April 2004
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Abstract In *Enterococcus hirae*, copper homeostasis is controlled by the *cop* operon, which encodes the copper-responsive repressor CopY, the copper chaperone CopZ, and two copper ATPases, CopA and CopB. The four genes are under control of CopY, which is a homodimeric zinc protein, $[Zn(II)CopY]_2$. It acts as a copper-responsive repressor: when media copper is raised, CopY is released from the DNA, allowing transcription to proceed. This involves the conversion of $[Zn(II)CopY]_2$ to $[Cu(I)_2CopY]_2$, which is no longer able to bind to the promoter. Binding analysis of $[Zn(II)CopY]_2$ to orthologous promoters and to control DNA by surface plasmon resonance analysis defined the consensus sequence TACAnnTGTA as the repressor binding element, or "*cop* box", of Gram-positive bacteria. Association and dissociation rates for the CopY–DNA interaction in the absence and presence of added copper were determined. The dissociation rate of $[Zn(II)CopY]_2$ from the promoter was 7.3×10^{-6} s^{-1} and was increased to 5×10^{-5} s^{-1} in the presence of copper. This copper-induced change may be the underlying mechanism of copper induction. Induction of the *cop* operon was also assessed in vivo with a biosensor containing a *lux* reporter system under the control of the *E. hirae cop* promoter. Half-maximal induction of this biosensor was observed at 5 μM media copper, which delineates the ambient copper concentration to which the *cop* operon responds in vivo.

Keywords Copper homeostasis · DNA–repressor interaction · Surface plasmon resonance · *Enterococcus hirae* · Gene regulation

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Abbreviations TCEP: tris(2-carboxyethyl)phosphine · RU: response units · TG: buffer 50 mM tris-SO_4, pH 7.8, 5% (v/v) glycerol

Introduction

Copper is fundamental to all living organisms, from bacteria to humans. While deficiency in copper is critical, excessive accumulation of the metal is toxic to cells. This makes it crucial for organisms to regulate the uptake, intracellular routing, and excretion of copper accurately. In the Gram-positive bacterium *E. hirae*, copper homeostasis is apparently controlled by the chromosomal *cop* operon. The operon consists of the four genes, *copY*, *copZ*, *copA*, and *copB*. *CopY* encodes a copper-responsive repressor, *copZ* encodes a copper chaperone, and *copA* and *copB* are the code for CPx-type membrane copper ATPases which are involved in copper uptake and secretion, respectively [1, 2, 3, 4].

The *cop* operon is under control of CopY, which acts as a copper-responsive repressor: at low ambient copper concentrations, the operon is repressed; in rich growth media, maximal induction of transcription is observed by media copper concentrations above 1 mM. CopY is a homodimeric zinc protein of the form $[Zn(II)CopY]_2$ [5]. It binds to two distinct sites of the promoter region, as shown by DNaseI footprinting. The two CopY binding sites feature an inverted repeat and flank the start of transcription (Fig. 1 [6, 7]). The N-terminal half of CopY shows 30% sequence similarity to the bacterial repressors of ß-lactamases, PenI, of *Bacillus licheniformis* [8] and related proteins and probably corresponds to the domain that recognizes the DNA promoter sequences [9]. The C-terminal region of CopY harbors a CxCxxxxCxC metal-binding motif. In its DNA-binding form, a zinc ion is complexed to the four cysteine residues. Upon induction of the *cop* operon by copper, two Cu(I) ions displace the zinc and provoke the release of CopY from the DNA [5, 10]. Copper is delivered to

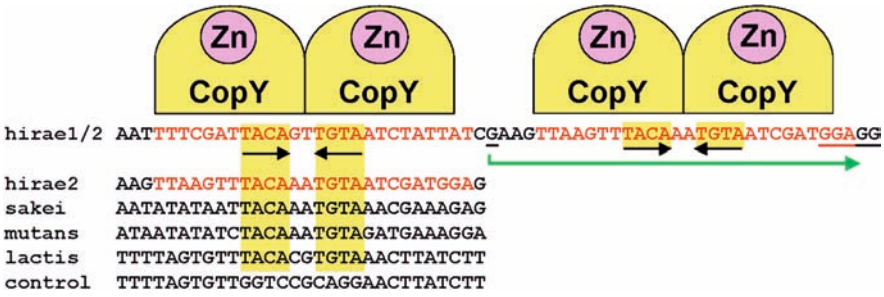

Fig. 1 Schematic representation of the $[Zn(II)CopY]_2$–DNA interaction. The conserved *cop* boxes are emphasized by *yellow rectangles* and the inverted repeat indicated by *black arrows*. A $[Zn(II)CopY]_2$ dimer binds to each of the two *cop* boxes of the *E. hirae* promoter (*hirae1/2*) and protects the regions indicated by red type from DNaseI digestion [6]. Transcription starts at the *underlined* G residue and is symbolized by the *green arrow*. Aligned below the first *E. hirae cop* box are the second *E. hirae cop* box (*hirae2*) and the *cop* boxes of *Lactobacillus sakei* (*sakei*), *Streptococcus mutans* (*mutans*), and *Lactococcus lactis* (*lactis*). Also shown is a control oligonucleotide (control) derived from the *L. lactis* promoter by mutation of the inverted repeat

CopY by the CopZ copper chaperone, a 69-amino acid protein belonging to the family of ubiquitous metallochaperones (cf. Fig. 8 [11, 12]).

Similar tripartite regulatory systems are also found in other lactic acid bacteria. The regulation of gene expression by copper is a key element of copper homeostasis, and the kinetic analysis of this regulation will help in the understanding of copper homeostasis. We used here, for the first time, surface plasmon resonance analysis to assess quantitatively kinetic parameters of the interaction of the *E. hirae* CopY repressor with the promoter. The in vitro findings were complemented by in vivo measurements with a *lux* reporter system under the control of the *E. hirae cop*-promoter/repressor/chaperone system in an *Escherichia coli* host deficient in copper homeostasis [13].

Materials and methods

Reagents

P-20 (ultra-pure Tween-20) was supplied by Biacore and tris(2-carboxyethyl)phosphate (TCEP) by Aldrich. All other chemicals were from Sigma Chemical Corp. (St. Louis, MO, USA) or from Merck (Darmstadt, Germany) and were of analytical grade. The following oligonucleotides were synthesized by Microsynth (Balgach, Switzerland): biotinylated *E. hirae* promoter (hirae1), 5′-AAGTTAAGTTTACAAATGTAATCGATGGAG; complementary strand, 5′-CTCCATCGATTACATTTGTAAACTTAACTT; biotinylated control (control), 5′-TTTTAGTGTTGGTCCGCAGGAACTTATCTT; complementary strand, 5′-AAGATAAGTTCCTGCGGACCAACACTAAAA; biotinylated *S. mutans* promoter (mutans), 5′-ATAATATATCTACAAATGTAGATGAAAGGA; complementary strand, 5′-TCCTTTCATCTACATTTGTAGATATATTAT; biotinylated *L. lactis* promoter (lactis), 5′-TTTTAGTGTTTACACGTGTAAACTTATCTT; complementary strand, 5′-AAGATAAGTTTACACGTGTAAACACTAAAA.

Construction of the CopY expression vector

The *copY* gene was amplified from plasmid pWY145 [6] with the primers 5′-GCTTGGATTCTCACCAATAAAA and 5′-GGATCCATGGAAGAAAAGAGAGTATTAATT and TaqPlus DNA polymerase (Stratagene). The product was cut with *BamHI* and *HindIII* and cloned into pQE8 (Qiagen), digested with the same enzymes. This resulted in the vector pWH6, expressing CopY with a hexa-histidine N-terminal tag. The correct DNA sequence of the clone was verified by sequencing.

Protein purification

$[Zn(II)CopY]_2$ is the native form of CopY isolated from *E. hirae* or *E. coli*. For its purification, BL21(DE3) cells (Stratagene) containing plasmid pWH6 were grown aerobically at 37 °C to an A_{550} of 0.4. Following induction with 1 mM isopropyl-1-thio-ß-D-galactopyranoside for 4 h, the cells were harvested by centrifugation for 10 min at 5,000 *g*. The cell pellet was washed twice with 200 ml TG buffer (50 mM tris-SO_4, pH 7.8, 5% (v/v) glycerol) and resuspended in 5 ml of TG buffer/g of wet cells. The cells were broken by three passages through a French press at 40 MPa. The cell debris was collected by centrifugation for 1 h at 90,000 *g* and the supernatant passed through a Ni-NTA Superflow (Qiagen) column. $[Zn(II)CopY]_2$ was eluted with TG buffer containing 200 mM imidazole. Final purification was achieved by gel filtration on a TSK3000G column in TG buffer. CopY without a histidine tag and CopZ were purified as previously described [6, 11]. Protein concentrations were determined by quantitative amino acid analysis. Zinc contents were assessed by inductively coupled plasma atomic emission and were, on average, 1.1 Zn per CopY monomer in purified $[Zn(II)CopY]_2$.

Copper loading of CopZ

CopZ at 587 µg/ml was dialyzed twice for 2 h at 4 °C against buffer Y (20 mM tris acetate, pH 8.0, 5 mM

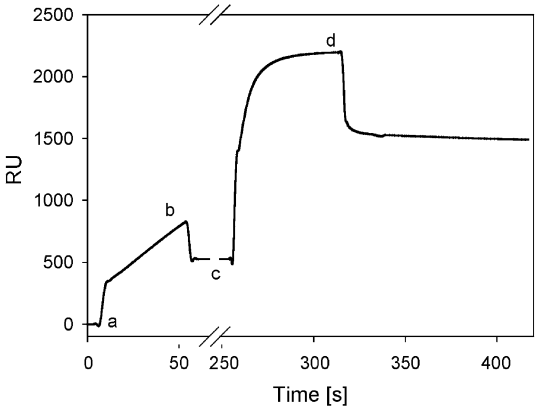

Fig. 2 Saturation of the promoter binding site with $[Zn(II)CopY]_2$. Injection of 2 μg/ml biotinylated hirae2-DNA onto the streptavidine-coated sensor chip was started at point *a* and continued to point *b*, followed by extensive washing with 2 M $MgSO_4$ (*break in the curve*). Injection of 100 μg/ml of *E. hirae* $[Zn(II)CopY]_2$ was started at point *c*, followed by injection of buffer, started at point *d* and continued to the end of the curve. Other details are outlined under Materials and methods

magnesium acetate, 50 mM sodium acetate, 1 mM calcium acetate, 2% acetonitrile, and 0.05% P-20). CopZ was then reduced for 15 min at room temperature by adding 1/10 volume of 50 mM TCEP in buffer Y. Cu(I)-acetonitrile, prepared as described by Hemmerich and Sigwart [14], was diluted 1,000-fold from 10 mM stock in 2% acetonitrile, 5 mM perchloric acid, into the CopZ solution, resulting in a final concentration of 10 μM Cu(I)-acetonitrile. Incubation was continued for an additional 30 min. Copper-loaded Cu(I)CopZ was diluted to the concentrations indicated.

Coupling of DNA to the sensor chip

For coupling, 500 μl of the biotinylated 29-mer oligonucleotide (1.25 μg/ml) was mixed with 500 μl of the complementary, non-biotinylated 29-mer oligonucleotide (1.5 μg/ml). The mix was heated to 80 °C and slowly cooled to 30 °C within 1 h. Avidin-containing SA sensor chips were conditioned with three consecutive 1-min injections of 1 M NaCl in 50 mM NaOH, followed by the injection of 2.5 μg/ml biotinylated oligonucleotide until binding of approximately 500 RU (response units) was achieved.

Coupling of CopY to the sensor chip

The surface of CM5 chips were activated by injecting 45 μl of *N*-hydroxylsuccinimide/*N*-hydroxylsuccinimide and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide at a flow rate of 5 μl/min. Protein at 2.5 μg/ml in 20 mM sodium acetate, pH 4.5, was injected at a flow rate of 10 μl/min until an increase of about 500 RU was reached. To quench excessive reactive groups, 30 μl of 1 M ethanolamine, pH 9.0, was injected. This was followed by washing with 2 M $MgSO_4$ to remove non-covalently bound protein.

Surface plasmon resonance analysis

Experiments were performed on a Biacore Upgrade instrument at 25 °C, using a flow rate of 5 μl/min. All runs were performed in buffer Y, and protein samples were dialyzed against this buffer before use. Between assays, sensor chips were reconditioned with two consecutive washes of 10 μl of 10 mM EDTA and 2 M $MgCl_2$. Data analysis was performed with BIAevaluation software version 3.1 (Biacore). Interactions were fitted by the Langmuir binding model ($A + B = AB$). The calculated association (k_a) and dissociation (k_d) rates were derived by fitting of the Biacore curves. Association and dissociation phases were fitted separately. K_D values were calculated from the relation $K_D = k_d/k_a$.

Construction of the *lux* reporter plasmid

The 3′-end of the *E. hirae cop* operon, containing the promoter and the *copY* and *copZ* genes, was cut out of pOA1 [3] with EcoRI and HindIII, made blunt-ended with Klenow DNA polymerase, and ligated into pC3 [15], cut with the same enzymes and also made blunt-ended with Klenow DNA polymerase. The resultant plasmid, pOC1, was cut with BamHI and PstI and ligated with the *luxCDABE* cluster excised from pUCD615 [16] with the same enzymes, resulting in plasmid pOCL.

Measurement of *lux* induction

The *E. coli* copA-knockout strain DW3110 [13] containing the *lux* reporter plasmid pOCL was grown aerobically at 37 °C to mid-log phase in 12 ml of LB media. Cells were then centrifuged at room temperature and the cell pellet resuspended in 24 ml of 0.9% NaCl and 1% glucose (salt media). Aliquots of 1 ml were transferred to Eppendorf tubes and induced at room temperature by the addition of $CuSO_4$. Emitted light was measured after 1 h (LB media) or 2 h (salt media) with an LAS-1000 CCD camera (Fuji Photo Film, Japan) for 10 min. The images were integrated with the AIDA software (Raytest Isotopenmessgeräte, Straubenhardt, Germany).

Results

It was shown previously by DNase footprinting and band-shift assays that there are two distinct binding sites for CopY in the promoter region of *E. hirae cop* operon [6]. By comparison of these regions with the promoter sites of the *cop* operons of *Lactococcus lactis* and *Streptococcus mutans*, we identified a conserved inverted

repeat or "*cop*-box" that is common to these promoters (Fig. 1). A control oligonucleotide with a randomly mutated *cop*-box did not exhibit significant binding of CopY (cf. Fig. 1, control). This suggests that the eight bases of the *cop*-box sequence represent the consensus repressor binding site.

To characterize the binding of *E. hirae* CopY in quantitative terms, biotinylated double-stranded *E. hirae* promoter DNA (hirae2) was coupled to a streptavidine-coated Biacore sensor chip, and the binding of $[Zn(II)CopY]_2$ to the DNA was measured by surface plasmon resonance analysis. Figure 2 shows the initial DNA loading of the sensor chip and subsequent $[Zn(II)CopY]_2$ binding and dissociation. At point *a*, injection of 2 μg/ml of hirae2-DNA was started and continued until 484 RU was reached at point *b*. (Note that the upward jump at *a* and the downward jump at *b* are due to the changes in refractive index of the buffers and not due to mass changes on the chip surface.) During the extensive wash with 2 M $MgCl_2$ (axis break), there was no apparent loss of DNA (the limit of detection was 10 RU). At point *c*, 100 μg/ml of CopY was injected until saturation of the binding to DNA, yielding 947 RU. Since the response units are directly proportional to the mass bound to the chip surface, the stoichiometry of the DNA–CopY complex can be calculated. The chip, containing 484 RU of biotinylated, double-stranded hirae2 oligonucleotide with an M_r of 18,651, bound 947 RU of $[Zn(II)CopY]_2$ with an M_r of 33,285. This corresponds to a calculated stoichiometry of 1.09 $[Zn(II)CopY]_2$ bound per oligonucleotide and shows that the model of one $[Zn(II)CopY]_2$ dimer binding to each *cop* box is correct. Since histidine-tagged CopY and native CopY worked equally well and exhibited essentially the same binding profiles, experiments were conducted with histidine-tagged CopY.

The CopY repressor was also cross-tested with promoter DNA of *Lactococcus lactis* and *Streptococcus mutans*. To these heterologous promoters, CopY showed the same binding activity as the endemic *E. hirae* promoter (not shown). This supports the hypothesis that the *cop* box, which is conserved in all these promoters, is the consensus repressor binding element. In line with this, there was no significant CopY binding to a control promoter in which the eight bases of the inverted repeat were randomly changed. In addition, there was no measurable interaction between CopY and a streptavidine chip without bound DNA, which shows that the repressor–DNA interaction was specific.

The association and dissociation rates of the interaction between CopY and the *cop* promoter were assessed by measuring the binding of $[Zn(II)CopY]_2$ to promoter DNA at different CopY concentrations (Fig. 3). The association and dissociation curves were fitted with the BIAevaluation software. This resulted in an association rate constant k_a of 4.3×10^4 M^{-1} s^{-1} and a dissociation rate k_d of 7.3×10^{-6} s^{-1}. Accordingly, the affinity of $[Zn(II)CopY]_2$ for the promoter could be calculated from $K_D = k_d/k_a$ as 1.7×10^{-10} M. In the

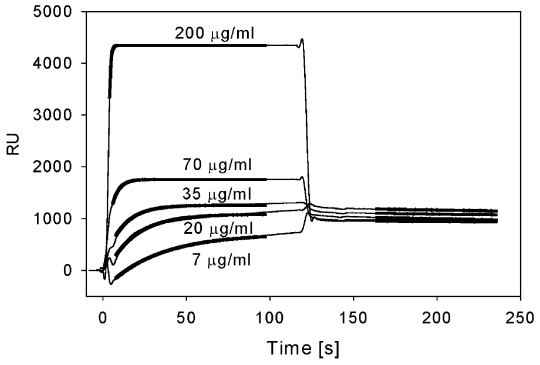

Fig. 3 Association and dissociation of CopY with the *E. hirae* promoter. $[Zn(II)CopY]_2$ was injected for 2 min at different concentrations and a flow rate of 10 μl/min. This was followed by the injection of buffer Y. Other details are given under Materials and methods

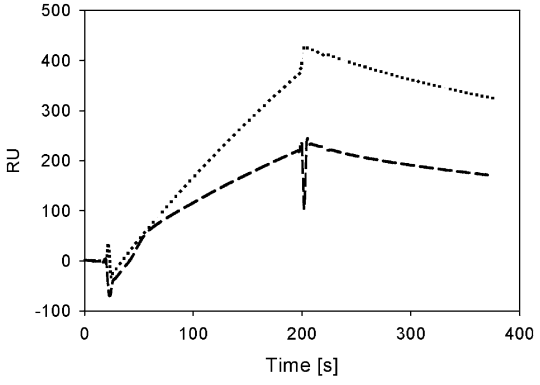

Fig. 4 Interactions of CopY with CopZ and Cu(I)CopZ. $[Zn(II)CopY]_2$ chemically cross-linked to a sensor chip was challenged for 3 min with 35 μg/ml of CopZ (----), and 35 μg/ml of Cu(I)CopZ (····), followed by the injection of buffer Y. Other details are as outlined under Materials and methods

presence of copper, CopY dissociated from the promoter as $[Cu(I)_2CopY]_2$ at a rate of 5×10^{-5} s^{-1}. An association rate for $[Cu(I)_2CopY]_2$ could not be determined due to the unavailability of this complex. Presumably, the observed changes in the affinity of CopY for the *cop*-box partake in induction of the *cop* operon by copper in vivo.

A possible interfering interaction of $[Zn(II)CopY]_2$ with itself was analyzed by direct coupling of $[Zn(II)CopY]_2$ to the chip surface, yielding approximately 500 RU. Injecting different concentrations of $[Zn(II)CopY]_2$ onto this chip did not reveal any significant interaction of CopY with itself (not shown). Using the same $[Zn(II)CopY]_2$ chip, the interaction with CopZ and copper-loaded Cu(I)CopZ was analyzed. Kinetic parameters were determined at CopZ and Cu(I)CopZ concentrations of 587, 353, 117, 59, and 35 μg/ml. (For clarity, only one concentration is shown in Fig. 4.) While the k_d values differed only two-fold between CopZ and Cu(I)CopZ, the k_a value for Cu(I)CopZ was 440-fold higher than that for CopZ (cf. Fig. 8). When

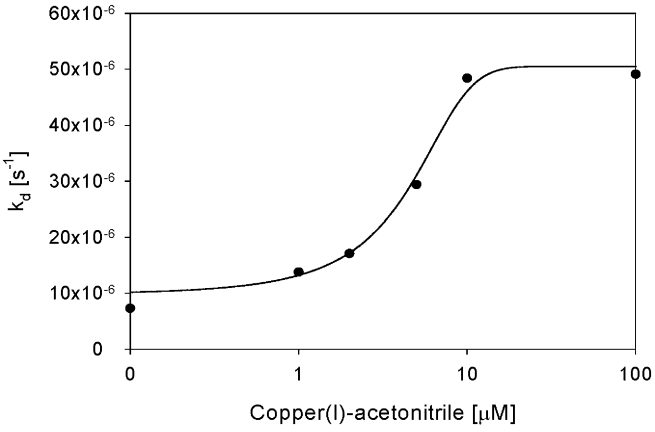

Fig. 5 Copper-dependence of the dissociation of CopY from the promoter. Dissociation rates of CopY from a sensor chip containing hirae2-DNA was measured at different copper(I)-acetonitrile concentrations, essentially as described in Fig. 3

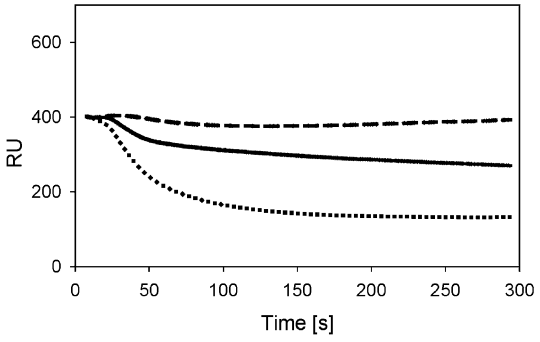

Fig. 6 Influence of CopZ on dissociation of CopY. CopY (950 RU) was bound to a hirae2-DNA-containing sensor chip, and CopY release was measured by injecting buffer Y (*solid curve*), 10 μg/ml of CopZ (*dashed curve*), or 10 μg/ml of Cu(I)CopZ (*dotted curve*). Other details are described under Materials and methods

CopZ was immobilized, no interaction with $[Zn(II)CopY]_2$ was detected (not shown). Most likely, this was due to steric hindrance. The coupling method we used preferentially crosslinks proteins via the ϵ-amino groups of lysine residues. There is a cluster of six lysine residues one side of CopZ, and we had shown previously by site-directed mutagenesis that these lysine residues are required for interaction with $[Zn(II)CopY]_2$ [10].

From previous in vivo and in vitro studies, it was clear that copper releases zinc from $[Zn(II)CopY]_2$, thereby releasing the repressor from the DNA and allowing transcription of the *cop* operon to proceed [5, 6, 7]. Thus, the effect of Cu(I) on the release of $[Zn(II)CopY]_2$ bound to a sensor chip with immobilized hirae1 DNA was investigated. CopY release was triggered with copper(I)-acetonitrile, a copper(I) complex which is stable even under aerobic conditions (Fig. 5). The dissociation rate of the $[Zn(II)CopY]_2$–DNA complex in the presence of 100 μM of the copper(I)-chelator bicinchoninic acid was 7.3×10^{-6} s^{-1}. This value represents a lower threshold for the dissociation rate of the $[Zn(II)CopY]_2$–DNA complex in the absence of copper. The rate of decomposition of the complex increased sevenfold in the presence of 10 μM copper(I)-acetonitrile, reaching a value of 5×10^{-5} s^{-1}. Presumably, this rate change is responsible for the induction process in vivo.

Inside the cell, the delivery of copper to CopY is supposed to be effected by Cu(I)CopZ. To investigate this step by surface plasmon resonance, $[Zn(II)CopY]_2$ was bound to a chip containing *cop* promoter DNA, and the release of CopY by Cu(I)CopZ was measured (Fig. 6). When CopZ was passed over the chip, there was a bi-phasic signal: a decrease in RU followed by an increase in RU. This was due to two reactions taking place simultaneously: the binding of CopZ to CopY, leading to an *increase* in RU, and the release of CopY from the chip, resulting in a *decrease* in RU. So binding of CopZ masks simultaneous release of CopY, which precluded the determination of kinetic constants for this tripartite reaction. When Cu(I)CopZ instead of CopZ was injected, the decrease in RU was strongly accelerated due to the accelerated release of CopY from the DNA chip. Qualitatively, this shows that Cu(I)CopZ accelerates the release of $[Zn(II)CopY]_2$ from the promoter, in line with the presumed in vivo function of Cu(I)CopZ in delivering copper to the $[Zn(II)CopY]_2$ repressor dimer.

The copper concentration necessary to induce the *cop* operon in vivo was assessed by constructing a biosensor based on the light-producing *lux* gene cluster of *Vibrio fischeri*. The plasmid pOCL contained the 3′ end of the *E. hirae cop* operon, consisting of the *cop* promoter, the *copY* repressor gene, and the copZ gene for the copper chaperone. Downstream of the copZ gene, the operon was fused to the *lux* gene cluster of *V. fischeri*, thus placing these genes under the control of the regulatory system of the *E. hirae cop* operon. The construct was transformed into the ΔcopA *Escherichia coli* strain DW3110. This strain is deficient in the copper-secreting CopA ATPase, the major, or possibly sole, extrusion system for cytoplasmic copper [17, 18]. The luminescence produced by the expression of the *lux* genes under the control of the *cop* promoter was measured as a function of ambient copper concentrations. In LB media, luminescence ensued at 300 μM added copper and reached a maximum at 2 mM added copper (Fig. 7). At higher copper concentrations, luminescence decreased due to copper toxicity, which is apparent in growth experiments (not shown).

In contrast to the response in LB media, luminescence measured in 0.9% NaCl, with 1% glucose as an energy source, already started at 2 μM added copper and reached a maximum at 10 μM. Clearly, in LB media, a large fraction of the added copper was complexed and thus not bioavailable. The copper levels required for induction in simple salt media cannot be translated into intracellular copper concentrations because of copper binding to intracellular constituents.

The experiment does, however, suggest the ambient copper concentration to which bacteria react by up-regulating the *cop* operon.

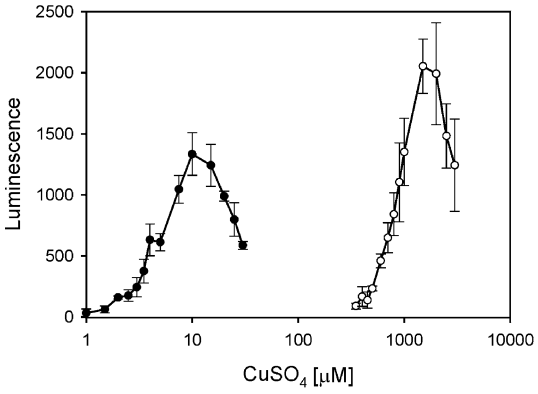

Fig. 7 In vivo induction of the *cop* operon measured with a *lux* biosensor. *E. coli* Δ*copA* cells containing the biosensor plasmid pOCL were exposed to copper in 0.9% NaCl, 1% glucose (*filled circles*), or in LB media (*open circles*). Luminescence was measured with a cooled CCD camera. Other details are given under Materials and methods

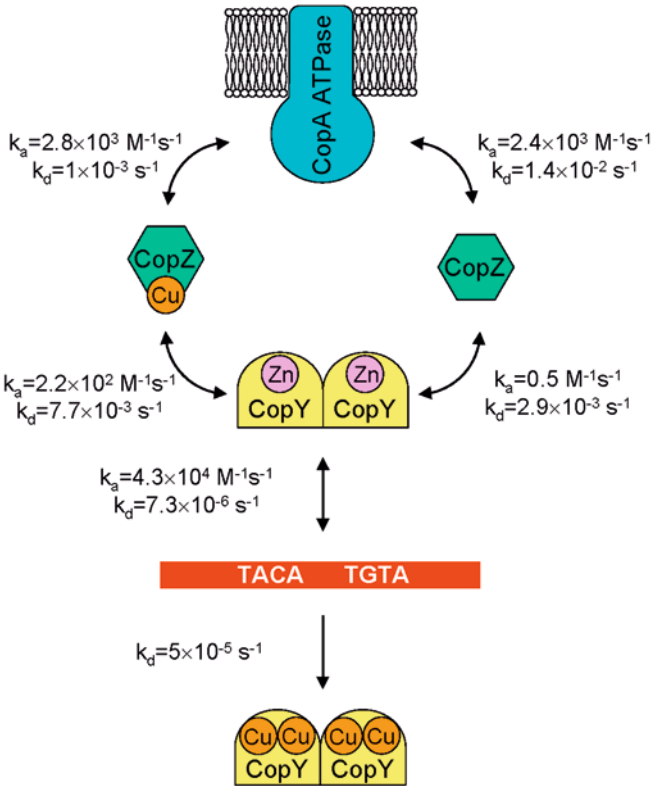

Fig. 8 Overview of all interaction kinetics between elements involved in copper homeostasis. Shown are the CopA copper ATPase of the plasma membrane (*blue*), the CopZ and Cu(I)CopZ chaperones (*green*), $CopY_2$ and $[Zn(II)CopY]_2$ repressor dimers (*yellow*), and the promoter region with the *cop* box (*red*). The kinetic values for the respective interactions are indicated. Values for the interaction of CopZ and Cu(I)CopZ with CopA were taken from reference [23]

Discussion

Related Gram-positive bacteria were found by database sequence-similarity searching to contain operons similar to the *cop* operon of *E. hirae*. Although some of these operons are missing the equivalent of the *E. hirae* copB or copZ genes, they do possess the genes encoding CopY-like repressors and CPx-type heavy-metal ATPases [4], which presumably are copper efflux pumps. Sequence comparison of the putative *cop* promoter/repressor binding regions in these organisms revealed a common motif, consisting of an inverted repeat of the sequence TACAnnTGTA. This motif forms a type of "*cop* box", which appears to be the binding site for CopY-like copper-responsive repressors. This is supported by three observations: (1) the motif is conserved in all operons related to the *E. hirae cop* operon, (2) *E. hirae* $[Zn(II)CopY]_2$ exhibited the same affinity for these heterologous promoter regions which we tested, and (3) mutation of the conserved inverted-repeat completely abolishes repressor binding. The binding site of the copper-responsive repressor of *E. coli*, CueR, does not share sequence similarity to the *cop* box, which may thus be specific for Gram-positive bacteria [19, 20].

We measured, for the first time, kinetics for copper-repressor–DNA interactions by surface plasmon resonance analysis. Since the signal changes (RU) in surface plasmon resonance analysis are proportional to the mass bound to the sensor chip, stoichiometries for the repressor–DNA interaction could be derived. Under the experimental conditions used, 1.09 $[Zn(II)CopY]_2$ per oligonucleotide could be bound. This is very close to the theoretical value of one, providing experimental support for the hypothesis that one $[Zn(II)CopY]_2$ dimer binds to a single *cop*-box. It also shows that essentially all the oligonucleotides on the sensor chip were accessible to $[Zn(II)CopY]_2$ under our experimental conditions.

We determined an equilibrium dissociation constant, K_D, for the $[Zn(II)CopY]_2$–DNA interaction of 1.7×10^{-10} in the absence of copper. The association rate of $[Zn(II)CopY]_2$ with the DNA target was not affected by copper. In contrast, the dissociation rate of the $[Zn(II)CopY]_2$–DNA complex was increased seven-fold in the presence of copper (Fig. 8). This increase is consistent with the in vivo action of CopY, i.e. the release from the DNA-binding site upon contact with copper and the formation of a $[Cu(I)_2CopY]_2$ complex. Copper-induced dissociation of CopY from the DNA had already qualitatively been shown by band-shift experiments [6]. The modulation of the affinity of a repressor for its DNA target by a chemical inducer has not been determined quantitatively before. The EthR repressor, a member of the TetR/CamR family of repressors, exhibited a K_D of only 1.5×10^{-7} M and showed strong cooperativity, but did not undergo a change in affinity by the presence of the purported inducer ethionamide [21]. The thermo-inducible *cts*-52 mutant repressor of *Bacillus subtilis* phage φ 105 had affinities for DNA

similar to those of CopY, namely in the range of 0.2–1×10^{-10}, depending on the promoter [22]. Raising the temperature from 37 to 50 °C led to a more than ten-fold decrease in the affinity of the repressor for some of the promoters, explaining thermo-induction [22]. Thus, relatively small changes in equilibrium-dissociation constants between the repressor and the DNA target appear to be sufficient to trigger induction of transcription.

The rate of dissociation of CopZ from $[Zn(II)CopY]_2$ differed only two-fold in the presence or absence of copper. In contrast, the association rate of Cu(I)CopZ with $[Zn(II)CopY]_2$ was 440-fold higher than that of CopZ. This supports the in vivo function of Cu(I)CopZ in delivering copper to $[Zn(II)CopY]_2$ [5, 10]. Once CopZ has donated the copper to CopY (and possibly other cellular constituents requiring copper), it has to be re-loaded with copper during the cyclic process of this chaperone. This presumably occurs at CopA [23]. The kinetic parameters of the interaction of CopZ and CopA had been determined earlier by surface plasmon resonance analysis [23] and corroborated the suggested CopZ cycle (Fig. 8). The association rates of CopZ and Cu(I)CopZ with the CopA ATPase were very similar, while the dissociation rate of Cu(I)CopZ was increased 14-fold compared to that of CopZ.

It was observed that copper(I)-acetonitrile in excess of 2 μM enhanced the dissociation of CopY from the DNA about five-fold. In the presence of 10 μM copper, the maximal dissociation rate of 5×10^{-5} s^{-1} was reached. Unfortunately, a dissociation constant for copper(I)-acetonitrile is not available. It is thus not possible to estimate the influence of the free copper concentration on the measured binding kinetics. For the copper-responsive activator of *E. coli*, CueR, the free copper concentration to which it responded to was estimated to be in the zeptomolar range (10^{-21} M [24]). This activator turns on copper homeostatic genes in response to copper, but the mechanism of this regulation does not resemble that of CopY of *E. hirae* [19, 20].

To determine the free ambient copper concentration to which the CopY repressor responds in vivo and to assess the validity of our in vitro Biacore data for the in vivo situation, a *lux* reporter system containing the *E. hirae cop* promoter and the genes for CopY and CopZ was used to determine the copper concentration required for induction of the *cop* operon. These experiments were carried out with an *E. coli* host deficient in CopA, the major and possibly only extrusion system for cytoplasmic copper [13, 18]. In LB media, half-maximal induction was observed in 1 mM copper. When the same measurement was performed in 0.9% NaCl, 1% glucose, this value shifted to 5 μM copper. This shift is due to copper-binding substances in LB medium which strongly chelate free copper. Copper binding by the salt media per se should be insignificant, but there could still be binding of copper to the cell envelope and other cellular constituents. We tried to avoid this as far as possible by working with dilute cell suspensions. At the cell concentrations used, further dilution of the cells did not measurably change the half-maximal induction level of 5 μM copper, suggesting that this is the relevant ambient copper concentration to activate transcription of the *cop* operon of *E. hirae*.

Acknowledgment We thank Kristian Raaby Poulsen and Thomas Weber for valuable experimental help and Christopher Rensing for providing the *E. coli* CopA-knockout strain DW3110. This work was supported by grant 31-68075.02 from the Swiss National Foundation, and by the International Copper Association (M.S.) and the Deutsche Forschungsgemeinschaft DFG (G.M.).

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