Structure–function analysis of purified *Enterococcus hirae* CopB copper ATPase: effect of Menkes/Wilson disease mutation homologues[1]

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The *Enterococcus hirae* CopB ATPase (EC 3.6.1.3) confers copper resistance to the organism by expelling excess copper. Two related human ATPase genes, *ATP7A* (EC 3.6.1.36) and *ATP7B* (EC 3.6.1.36), have been cloned as the loci of mutations causing Menkes and Wilson diseases, diseases of copper metabolism. Many mutations in these genes have been identified in patients. Since it has not yet been possible to purify the human copper ATPases, it has proved difficult to test the impact of mutations on ATPase function. Some mutations occur in highly conserved sequence motifs, suggesting that their effect on function can be tested with a homologous enzyme. Here, we used the *E. hirae* CopB ATPase to investigate the impact of such mutations on enzyme function *in vivo* and *in vitro*. The Menkes disease mutation of Cys-1000 → Arg, changing the conserved Cys-Pro-Cys ('CPC') motif, was mimicked in CopB. The corresponding Cys-396 → Ser CopB ATPase was unable to restore copper resistance in a CopB knock-out mutant *in vivo*. The purified mutant ATPase still formed an acylphosphate intermediate, but possessed no detectable ATP hydrolytic activity. The most frequent Wilson disease mutation, His-1069 → Gln, was introduced into CopB as His-480 → Gln (H480Q). This mutant CopB also failed to confer copper resistance to a CopB knock-out strain. Purified H480Q CopB formed an acylphosphate intermediate and retained a small, but significant, ATPase activity. Our results reveal that Cys-396 and His-480 of CopB are key residues for ATPase function, and similar roles are suggested for Cys-1000 and His-1069 of Menkes and Wilson ATPases respectively.

Key words: copper homoeostasis, CPx-type ATPase, genetic disease, humans, phosphorylation.

INTRODUCTION

Copper is an essential trace element that is required in every living organism. It serves as a cofactor in redox reactions, such as those catalysed by cytochrome *c* oxidase, superoxide dismutase, lysyl oxidase and dopamine β-hydroxylase. Excessive copper accumulation, however, can be toxic to cells, leading to oxidative damage of biomolecules. This makes the control of copper levels by homoeostatic mechanisms a cellular requirement.

The discovery of highly similar copper-transporting ATPases in humans and bacteria less than a decade ago has greatly advanced the understanding of copper homoeostasis. It is now evident that copper ATPases have a key role in the regulation of cellular copper and are present in most, if not all, cells throughout Nature [1,2]. The copper ATPases are distinct from classical P-type ATPases (Ca^{2+}-ATPase, Na^+,K^+-ATPase) in membrane topology and primary structure features. They thus form a subclass of the P-type ATPase family that has been designated as P_1-type ATPases, or CPx-type ATPases on the basis of a distinctive intramembranous 'CPC' (Cys-Pro-Cys) or 'CPH' (Cys-Pro-His) motif [3,4]. Figure 1 highlights the topological differences between a heavy-metal CPx-type ATPase and the Ca^{2+}-ATPase of the sarcoplasmic reticulum, for which a three-dimensional structure has recently become available [5]. The list of heavy-metal CPx-type ATPases is rapidly growing, and transport specificities now include silver, zinc, cadmium and lead [6–9].

The two human CPx-type copper ATPases that have been cloned as the genes mutated in Menkes and Wilson disease are receiving particular attention, in view of their role in health [10,11]. The two ATPases, also called ATP7A and ATP7B, exhibit around 60 % amino acid sequence identity, and are both localized primarily in a *trans*-Golgi compartment [12,13]. However, the Menkes and Wilson ATPases have different tissue distributions, and mutations of the corresponding genes give rise to entirely different diseases. Mutations in the Menkes gene are associated with a systemic copper deficiency, which is mainly caused by a low copper uptake through the intestine. Major hallmarks of the disease are severe neurological retardation and connective-tissue defects [14]. The spectrum of mutations underlying Menkes disease includes a wide range of different point mutations, but also extensive gene deletions [15]. Whereas the frequency of the X-chromosome-linked Menkes disease worldwide is only 1 in 300000, the autosomal-recessive Wilson disease occurs at a rate of about 1 in 30000. The disease is associated with a systemic overload of copper caused by a lack of hepatic clearance of the trace element. Major clinical manifestations of Wilson disease are neurological impairment and hepatic cirrhosis. In Wilson disease, more than 100 point mutations have been identified, but a single mutation, His-1069 → Gln, accounts for 30–40 % of the patients in North America and Northern Europe [16,17]. This mutation affects a His-Pro dipeptide motif (HP motif), which is conserved in all CPx-type ATPases, but is absent in other P-type ATPases [3].

Although diagnosis of Menkes and Wilson diseases by molecular genetics is now possible, correlations between particular mutations, severity of the diseases and prognoses are still elusive. In part, this is due to the unavailability of purified human copper ATPases for direct functional testing. In contrast, the CopB

Abbreviations used: CCD, charge-coupled-device; C1000R, C396S, H1069Q and H480Q, Cys-1000 → Arg, Cys-396 → Ser, His-1069 → Gln and His-480 → Gln site-directed mutations respectively; Ni-NTA, Ni^{2+}-nitrilotriacetate.

[1] This paper is dedicated to the memory of Walter Beuggert, with appreciation for his contribution to research.

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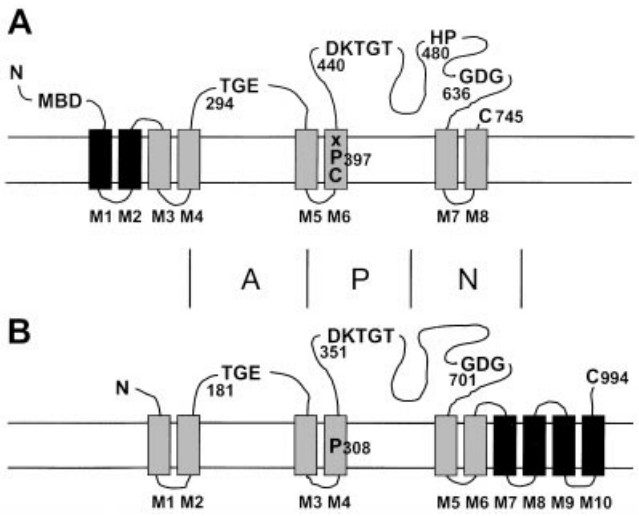

Figure 1 Comparison of the membrane topology of a CPx-type ATPase (A) and a non-heavy metal ATPase (B)

Shown are CopB (**A**) of *E. hirae* and the Ca^{2+}-ATPase of sarcoplasmic reticulum (**B**). Membrane helices are numbered M1 to M10. Helices common to both type of ATPases are in grey and helices unique to one type of ATPase are in black. Key sequence motifs are indicated in the one-letter amino acid code and the numbers denote the position of residues in the sequence. In the centre of the Figure, the approximate locations of the three cytoplasmic domains A, P and N are indicated (see the text for further details). MBD, metal-binding domain containing repeat metal binding sites; TGE, conserved site in transduction domain A; CPx, putative copper-binding site; DKTGT, phosphorylation site in domain P; HP, motif of unknown function, probably in domain N; GDG, nucleotide-binding site residues in domain N.

copper ATPase of *Enterococcus hirae* can readily be purified. Since key functional domains are highly conserved in all CPx-type ATPases, it is possible to use CopB as a model system to assess both *in vivo* and *in vitro* the functional impact of Menkes/Wilson disease mutations. CopB confers copper resistance to *E. hirae* by exporting copper under high-copper conditions. Copper transport by CopB has been demonstrated in membrane vesicles, and the enzyme has been purified to homogeneity, allowing direct measurement of enzymic parameters such as acylphosphate formation and ATP hydrolysis [18,19].

In the present study, we examined the two most characteristic conserved domains of CPx-type ATPases, the intramembranous CPx motif and the conserved HP motif, by site-directed mutagenesis of CopB. Mutant CopB ATPases were expressed in a CopB knock-out strain of *E. hirae*. Mutations in the CPx and the HP motif rendered the ATPase non-functional *in vivo*. Analysis of the purified enzymes *in vitro* showed that partial activities were retained by both mutant enzymes, shedding light on the functional significance of the corresponding conserved residues.

EXPERIMENTAL

Materials

Na_2ATP was supplied by Sigma Chemical Corp. (St. Louis, MO, U.S.A.), and [γ-^{32}P]ATP (3000 Ci/mmol) was from Amersham Pharmacia Biotech (Piscataway, NJ, U.S.A.). Dodecyl β-D-maltoside was purchased from Calbiochem–Novabiochem Corp. (San Diego, CA, U.S.A.). Trypticase peptone and yeast extract were from Difco Laboratories (Detroit, MI, U.S.A.). All other chemicals were from Merck (Darmstadt, Germany) or from Sigma, and were of analytical grade. Wild-type *E. hirae* (A.T.C.C.9790) was obtained from American Type Culture Collection and the Δ*cop*AB-null mutant has been described previously [20]. For all growth experiments, exponentially growing cells that were frozen at −70 °C in 17 % (v/v) glycerol were used for inoculation.

Plasmid construction

Plasmid pOB11 was generated by removing the *Nco*I site from the chloramphenicol resistance gene by partial digestion of pOB1 [21] with *Nco*I, followed by Klenow polymerase treatment and self-ligation. Mutations Cys-396 → Ser and His-480 → Gln (C396S and H480Q respectively) were introduced into the *copB* gene, cloned into the *Escherichia coli* vector pGEX 2T (Amersham Pharmacia Biotech), with the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, U.S.A.), using primers 5′-CGTGTTCATCATTGCTTCTCCGCATGC-3′ and 5′-GCATGCGGAGAAGCAATGATGAACACG-3′ to generate the C396S mutation, and primers 5′-GCACATG-CAAATCAACCTTTAGCGATTGG-3′ and 5′-CCAATCGC-TAAAGGTTGATTTGCATGTGC-3′ to generate the H480Q mutation. To obtain the corresponding *E. hirae* expression plasmids, pOB396 and pOB480, the mutated 2056 bp *Nco*I–*Sal*I fragment of *copB* was ligated into pOB11, cut with the same enzymes. The mutations were verified by sequencing the entire *copB* gene (Microsynth GmbH, Balgach, Switzerland). Plasmids were introduced into *E. hirae* by electroporation [22]. Standard molecular biology methods were conducted by following published procedures [23].

Growth experiments

Bacteria from cells frozen at the exponential stage of growth were streaked out on to N-media plates [1 % $Na_2HPO_4 \cdot 2H_2O$, 1 % (w/v) trypticase peptone, 0.5 % (w/v) yeast extract, 1 % (w/v) glucose and 1.5 % (w/v) agar] and incubated at 37 °C overnight. Plates were then replica-plated to plates containing the indicated concentrations of $CuSO_4$. Photographs of the plates were taken with an LAS-1000 charge-coupled-device (CCD) camera (Fuji Photo Film Co., Tokyo, Japan). To measure growth in liquid cultures and to determine CopB expression levels, 50 ml of N-media was inoculated with 200 μl of cells frozen at the exponential stage of growth, and then grown semi-anaerobically (in closed tubes, but not de-aerated) to the late-exponential growth phase at 37 °C in the presence of 4 mM $CuSO_4$. D_{546} values were determined on a Lambda 16 Spectrophotometer (PerkinElmer, Norwalk, CT, U.S.A.).

Purification of CopB

Cells were grown in 16 litres of N-media in a fermenter to D_{546} 0.5–0.7, induced with 0.1 mM of *o*-phenanthroline for 1 h, and then collected by centrifugation for 10 min at 8000 ***g***. The subsequent steps for the preparation of membranes from *E. hirae* were carried out as described previously [19]. Batches of 1 g of membrane protein were extracted with 1 g of dodecyl β-D-maltoside in 90 ml of buffer G [20 mM Tris/H_2SO_4 (pH 7.5)/5 mM $MgSO_4$/25 mM Na_2SO_4/1 mM 2-mercaptoethanol/1 μM $CuSO_4$/20 % (v/v) glycerol] supplemented with a 1/50 vol. of a protease inhibitor cocktail [19]; these were then incubated on ice for 90 min with constant stirring. The suspension was centrifuged at 90000 ***g*** for 45 min, and the supernatant was loaded on to a 2 cm × 4 cm Ni^{2+}-nitrilotriacetate (Ni-NTA) column (Qiagen), followed by elution

with 32 ml of a linear 0–100 mM imidazole gradient in G buffer without Na_2SO_4, but containing 0.05 % (w/v) dodecyl β-D-maltoside. CopB was eluted from the nickel column, and was purified further by absorption on an anion-exchange chromatography column (MonoQ 5/5; Amersham Pharmacia Biotech) that was eluted with 22 ml of a linear 0–60 mM Na_2SO_4 gradient in the same buffer, essentially as described previously [19]. The protein content of the purified fractions was determined by the method of Bradford [24] using BSA as the standard.

Western blotting

Cells from growth experiments were either scraped off culture dishes or harvested from 1 ml of culture. All samples were adjusted to correspond to approx. 10^9 cells, and were centrifuged for 1 min at 6000 ***g***. Cell pellets were dissolved in 50 μl of 10 mg/ml lysozyme, 1 mM EDTA and 10 mM Tris/HCl, pH 8. After incubation at room temperature for 10 min, the solution was frozen at -70 °C, followed by thawing, the addition of 10 μl of DNase I (at a concentration of 1 mg/ml in 100 mM $MgCl_2$), and further incubation for 5 min at room temperature. Samples were then denatured for 10 min at 37 °C in sample buffer and resolved by SDS/PAGE, as described previously [25]. Gels were subjected to Western blotting as described in [26], and were developed with a polyclonal antibody raised against CopB [20] and a horseradish-peroxidase-coupled donkey anti-(rabbit IgG) secondary antibody (Pierce Chemical Company, Rockford, IL, U.S.A.). Chemiluminescence generated with the ECL® kit (Amersham Pharmacia Biotech) was captured and quantified with an LAS-1000 CCD camera (Fuji Photo Film Co.) and the AIDA software (raytest Isotopenmessgeräte GmbH, Straubenhardt, Germany), respectively.

ATPase assay

Purified CopB (usually 1.1 μg) was preincubated for 10 min at 37 °C in assay buffer [50 mM Mes (pH 5.5)/50 mM KCl/5 mM $MgSO_4$/0.05 % (w/v) dodecyl-β-D-maltoside] in a total volume of 200 μl. The reaction was initiated by the addition of ATP, and samples were taken at 0, 10, 20 and 30 min and transferred to tubes containing 10 μl of 0.5 M sodium/EDTA at room temperature to stop the reaction. Phosphate released from ATP was subsequently determined with a colorimetric assay [27].

Detection of acylphosphate intermediates

Purified wild-type or mutant CopB (2.8–5 μg) was preincubated for 10 min at 37 °C in 100 μl of assay buffer (see above). The reaction was started by the addition of 17 nM (5 μCi) [γ-^{32}P]ATP. After 0.5, 2 or 8 min, 400 μl of 10 % (w/v) ice-cold trichloroacetic acid containing 1 mM sodium phosphate was added. Following 10 min on ice, the protein precipitate was centrifuged and the resultant pellet was washed with 0.5 ml of ice-cold water. The final pellet was dissolved in 30 μl of acidic sample buffer [0.5 mM sucrose/10 mM dithiothreitol/5 % (w/v) SDS/0.005 % Bromophenol Blue/50 mM sodium phosphate (pH 6)] and resolved on an acidic gel [28]. The gel was stained for 30 min in 0.2 % (w/v) Coomassie Brilliant Blue, 7.5 % (v/v) acetic acid and 50 % (v/v) methanol, followed by three washes in 20 % (v/v) ethanol/10 % (v/v) acetic acid (40 min each wash). Gels were photographed with a CCD camera, and staining intensities were evaluated with the AIDA software. After drying, the gel was exposed to a phosphoimaging screen for about 15 h and the screen was scanned with a PhosphorImager and evaluated by the ImageQuant software (Molecular Dynamics, Sunnyvale, CA, U.S.A.). All measurements were repeated at least three times.

RESULTS

CopB of *E. hirae* is involved in copper extrusion from the cell. Thus growth of cells with a deletion of CopB is inhibited by copper above 0.1 mM, whereas wild-type cells are unaffected by 4 mM copper. This offers a test for the function *in vivo* of CopB and the effects of mutations on its function. Two amino acids of great functional interest were chosen for analysis: the cysteine of the conserved CPx domain in the sixth transmembrane helix, and the conserved HP motif 40 residues C-terminal of the phosphorylated aspartic acid residue (Figure 1). The Menkes disease mutation Cys-1000 → Arg (C1000R), changing the conserved CPC motif to Arg-Pro-Cys, has been described as causing a severe phenotype, although with a long survival [29]. A corresponding mutation, C396S, was introduced into CopB. Cysteine was mutated to serine rather than to arginine to avoid structural disturbance that might ensue from incorporation of a comparatively large arginine side chain. The most frequent Wilson mutation His-1069 → Gln (H1069Q) was introduced in CopB as a corresponding H480Q mutation. Plasmids mutated by site-directed mutagenesis were transformed into a Δ*cop*AB strain to test for function *in vivo* by complementation of the copper-sensitive phenotype of this strain. The double-deletion Δ*cop*AB strain has a copper-sensitive phenotype indistinguishable from that of a Δ*cop*B strain, but can be transformed more efficiently [20].

Figure 2 shows that the Δ*cop*AB strain complemented with wild-type *cop*B on a plasmid grew well on plates containing 4 mM $CuSO_4$. In contrast, growth was not observed when the Δ*cop*AB strain was either not transformed or transformed with mutant C396S or H480Q CopB. Since both mutant ATPases were expressed, at least at the wild-type level (see below), the lack of function *in vivo* suggests that both mutations abolish copper transport by CopB.

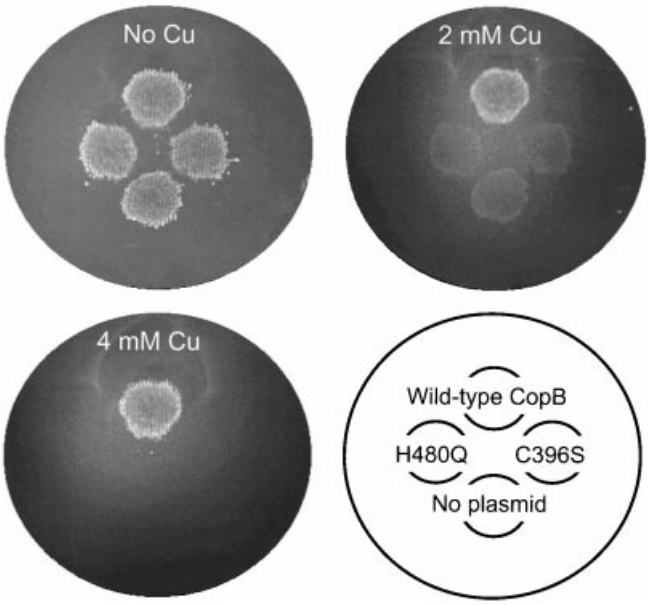

Figure 2 Replica plating of strains expressing wild-type and mutant CopB proteins

A Δ*cop*AB knock-out strain not complemented ('No plasmid') or complemented with wild-type or mutant CopB, as indicated on the Figure, was replicated to N-media plates containing the indicated amounts of copper. Plates were photographed after incubation for 10 h at 37 °C.

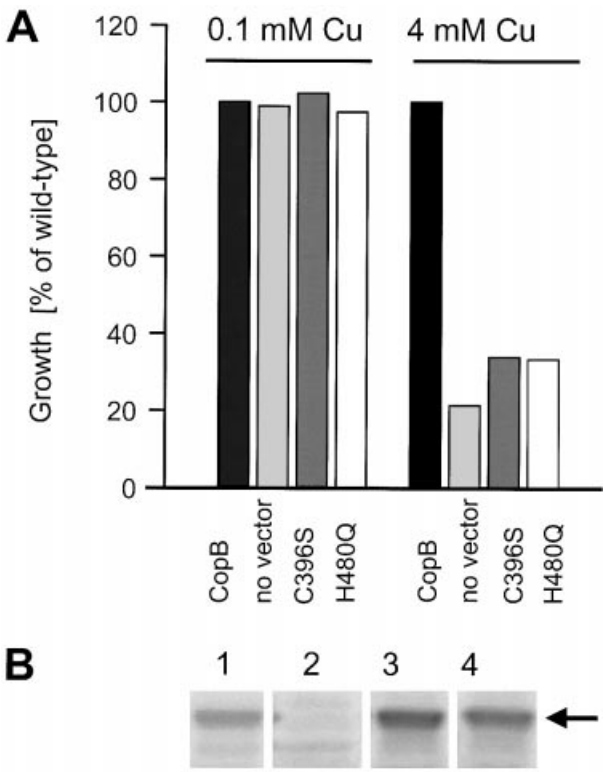

Figure 3 Growth in liquid cultures and CopB expression levels

Δ*cop*AB strains were transformed with plasmids, as indicated in the Figure. (**A**) Cell densities in liquid cultures grown for 14 h in N-media containing 0.1 mM or 4 mM $CuSO_4$ were measured photometrically. Final cell densities (= 100%) of cells expressing wild-type CopB (CopB) were 1.78 and 0.53 D_{545} in 0.1 and 4 mM copper respectively. Results are the means for three experiments. (**B**) Western blot of the strains used in (**A**). Lane 1, Δ*cop*AB expressing wild-type CopB; lane 2, untransformed Δ*cop*AB; lane 3, Δ*cop*AB expressing C396S; lane 4, Δ*cop*AB expressing H480Q. The arrow indicates the position of CopB.

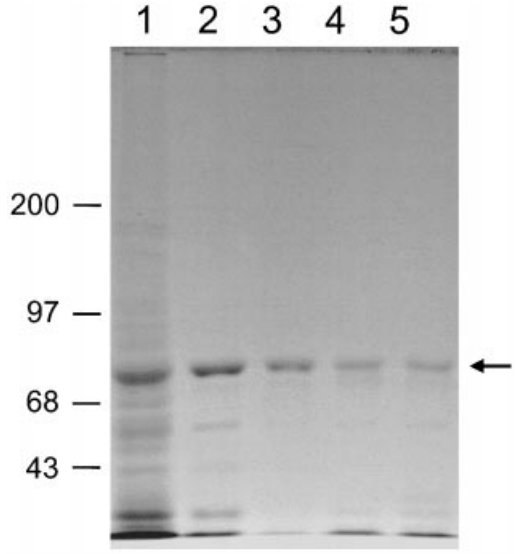

Figure 4 Purification of CopB

Lane 1, 50 μg of protein from *E. hirae* membrane extract; lane 2, 2 μg of CopB from the peak fraction of the Ni-NTA column; lane 3–5, 1 μg each of final purified wild-type CopB, C396S mutant CopB and H480Q mutant CopB respectively. Samples were resolved by SDS/7.5% (w/v) PAGE, and stained with Coomassie Brilliant Blue. The arrow indicates the position of CopB, and the scale on the left shows the migration of molecular-mass markers of the corresponding size in kDa.

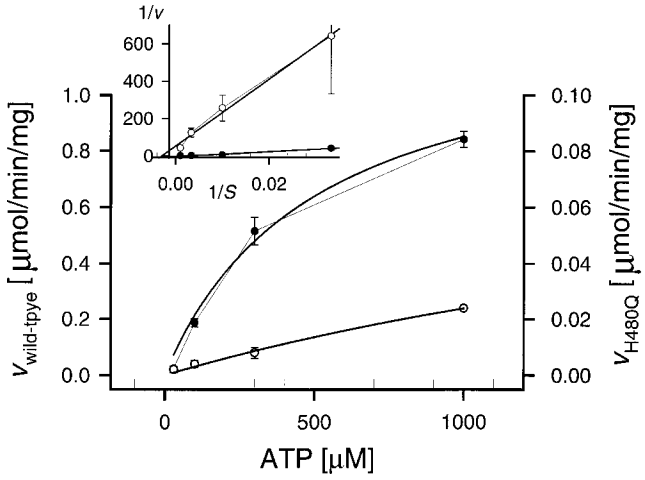

Figure 5 Enzyme kinetics of wild-type and H480Q CopB

Initial ATP-hydrolysis rates were determined for 10 min at 37° as a function of the ATP concentration of wild-type CopB (●, $v_{wild-type}$) and H480Q CopB (○, v_{H480Q}). Note the different *y*-axis scales for wild-type and H480Q CopB. The inset shows the data replotted according to the method of Lineweaver–Burk. The means ± S.D. for three experiments are shown. Details of the measurements are as described in the Experimental section.

The investigation of copper resistance in long-term liquid cultures gave similar results (Figure 3A). In the presence of 0.1 mM copper, all the strains grew essentially by the same extent. In 4 mM copper, cells expressing the two mutant ATPases displayed an end-stage of growth only 10% more than that of cells not expressing CopB. To ensure that lack of growth was not caused by a failure in CopB expression, the strains were analysed by Western blotting with an antibody raised against CopB (Figure 3B). Mutant CopB ATPases were expressed slightly better than that of the wild-type, showing that the failure of mutants C396S and H480Q to promote significant growth in 4 mM copper was not a result of reduced expression. The slightly higher growth yields of the two mutants compared with the untransformed Δ*cop*AB strain was due to very slow, residual growth, conceivably made possible by stoichiometric copper binding by catalytically inactive CopB. This would provide some buffering against toxic copper, similar to toxic-metal buffering by metallothioneins.

For the biochemical analysis of mutant CopB ATPases, they were overexpressed in *E. hirae* and purified by successive affinity chromatography on Ni-NTA, followed by anion-exchange chromatography. Figure 4 shows the progressive purification for wild-type CopB and the purity of wild-type and mutant CopB ATPases used for the studies *in vitro*. ATPase activity was assessed as a test for the overall function of the enzymes. The specific activity of wild-type CopB averaged 1.4 ± 0.31 μmol/min per mg (mean ± S.D.). Mutant C396S CopB had no detectable ATPase activity, whereas mutant H480Q CopB exhibited residual activity. This activity was not due to contaminating proteins, because the same trace contaminants were present in the inactive C396S CopB preparation, and H480Q preparations of various purities all exhibited the same residual activity.

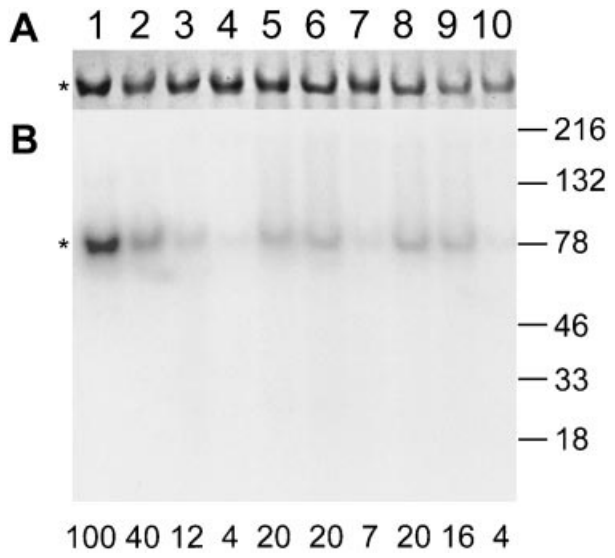

Figure 6 Acylphosphate formation by wild-type and mutant CopB

Purified protein was phosphorylated with [γ-^{32}P]ATP, resolved on an acidic SDS/7.5% polyacrylamide gel and stained with Coomassie Brilliant Blue (**A**), followed by autoradiography (**B**). Lanes 1–3, wild-type CopB labelled for 0.5, 2 and 8 min respectively; lane 4, wild-type CopB labelled for 0.5 min, followed by a 0.5 min chase with non-labelled ATP; lanes 5 and 6, C396S CopB labelled for 0.5 and 8 min respectively; lane 7, C396S CopB labelled for 0.5 min, followed by a 0.5 min chase with non-labelled ATP; lanes 8 and 9, H480Q CopB labelled for 0.5 and 8 min respectively; lane 10, H480Q CopB labelled for 0.5 min, followed by a 0.5 min chase with non-labelled ATP. The numbers at the bottom of the lanes indicate the relative [^{32}P]P_i incorporation, quantified by phosphoimaging and normalized with respect to the protein content of the bands. The asterisks mark the bands corresponding to CopB, and the scale on the right indicates the migration of molecular-mass standards of the corresponding sizes in kDa. Additional details of the procedure are as described in the Experimental section.

To obtain a more detailed picture of the effect caused by the H480Q mutation, enzyme kinetic parameters were assessed (Figure 5). Wild-type CopB exhibited a K_m for ATP of 0.5 ± 0.2 mM and a V_{max} of 1.3 ± 0.3 μmol/min per mg. For the mutant H480Q, K_m and V_{max} could not reliably be determined owing to the low activity, and it was not clear whether Michaelis–Menten kinetics were obeyed (the following numerical values were obtained from a preliminary regression analysis of the Lineweaver–Burk plot: K_m 0.4 ± 5.6 mM; V_{max} 0.02 ± 0.1 μmol/min per mg). These data suggest that the H480Q mutation affects primarily ATPase turnover, rather than ATP binding, but this requires verification.

A hallmark of P-type ATPases is the formation of an acylphosphate intermediate. The γ-phosphate of ATP forms a covalent bond with the aspartic acid residue of the conserved Asp-Lys-Thr-Gly-Thr motif of the protein. The energy of this high-energy bond is thought to be used to drive a subsequent step in the pumping cycle, resulting in the release of the phosphate as P_i. If the site of ATP binding and hydrolysis in CopB is unaffected by a mutation, we would expect acylphosphate formation to proceed, even in an enzyme that is transport-incompetent. Figure 6 shows acylphosphate formation by wild-type and mutant CopB ATPases. Maximal phosphorylation was attained in 0.5 min in wild-type and mutant CopB enzymes, but the extent of acylphosphate formation was reduced by 80% in both the C396S and the H480Q mutants. The acylphosphate could be 'chased' with non-labelled ATP, even in the mutant enzymes. This shows that the capacity for enzyme turnover is not entirely lost, even in the C396S mutant, which does not catalyse detectable ATP hydrolysis. In wild-type CopB, prolonged reaction times led to a diminished intensity of the acylphosphate band resulting from the depletion of the ATP by hydrolysis.

Taken together, it was found that the conserved CPx and HP motifs are key elements for copper ATPase function. Mutation of the corresponding cysteine and histidine residues led to non-functional phenotypes *in vivo*. *In vitro*, different partial reactions were still catalysed by the mutant enzymes, allowing a first delineation of the function of these conserved motifs. Mutation of the corresponding residues in Menkes and Wilson ATPases are expected to affect the function of these enzymes similarly.

DISCUSSION

Using the CopB copper ATPase, we analysed two conserved motifs of CPx-type ATPases, the CPx motif and the HP motif, using functional complementation *in vivo* and *in vitro* biochemical analysis of purified CopB. Both motifs are of particular interest because they are specific to heavy-metal ATPases and are not present in non-heavy-metal ATPases such as the Ca^{2+}- or Na^+,K^+-ATPases.

The CPx motif, which is CPC in Menkes and Wilson ATPase and CPH in CopB, has been postulated to be part of the ion channel through the membrane, chiefly on the basis of site-directed mutagenesis studies with the Ca^{2+}-ATPase [3,30]. With the recent advent of a three-dimensional structure for the Ca^{2+}-ATPase of sarcoplasmic reticulum, the role of this domain in ion binding is now clear [5]. In the Ca^{2+}-ATPase, the residues corresponding to the CPC motif (shown in bold) are part of the sequence Val^{304}-Ala-Ala-**Ile-Pro-Glu**^{309}. Owing to the different membrane topology of heavy-metal and non-heavy-metal ATPases, these residues are located in helix M6 in copper ATPases and in helix M4 in the calcium ATPase (cf. Figure 1). In the calcium ATPase, the type II calcium-binding site is primarily composed of residues in helix M4. The main-chain carbonyl oxygen atoms of Val-304, Ala-305 and Ile-307, and a side-chain oxygen atom of Glu-309 (plus oxygen atoms from Asn-796 and Asp-800), contribute to the site, requiring some unwinding of helix M4.

Since nitrogen and sulphur atoms are much better copper ligands than oxygen, it is apt that the residues corresponding to Ile-307 and Glu-309 are cysteine or histidine residues in heavy-metal ATPases. In agreement with the proposed key role of the CPx motif in ATPase function, the C396S mutation of CopB showed no function *in vivo* and the purified enzyme had no detectable ATPase activity. Direct binding measurements would be required to demonstrate the involvement of C396 in high-affinity copper binding. However, preliminary studies in our laboratory have indicated that the binding affinity of CopB for copper is in the low nanomolar range. Current instrumentation does not allow us to measure such low copper concentrations. A detailed understanding of the CPx motif in copper binding and translocation will thus have to await further technical developments.

Since ion translocation and ATP hydrolysis are coupled processes in an ion pump, a severe defect in ion translocation is expected to impair ATP hydrolytic activity, and vice versa. The C396S mutation still exhibited a reduced level of phosphoenzyme formation from ATP. This would suggest that the ATP-binding site and the phosphorylation site are not directly affected by this mutation. This could not be confirmed directly, because of undetectably low ATP hydrolytic activity. If the structure of a copper ATPase resembles that of the calcium ATPase, then metal binding, ATP binding and phosphorylation would occur

on distinct, widely separated domains [5]. A putative Menkes disease-causing mutation in the CPx-motif, C1000R, has been reported to cause a severe phenotype [15,29]. On the grounds discussed above, such a mutation would be expected to lead to a non-functional Menkes ATPase, and thus cause Menkes disease.

The data obtained with the CopB mutants are in agreement with experiments in which mutant proteins were used to complement *Saccharomyces cerevisiae* deficient in Ccc2p copper ATPase. This ATPase has a *trans*-Golgi localization analogous to that of the human copper ATPases, and is required for the incorporation of copper into Fet3p, a multi-copper oxidase required for high-affinity iron accumulation [31,32]. *Ccc2* knock-out strains are therefore growth-deficient on iron-depleted media, but can be functionally complemented by CCC2 homologues, such as the Menkes or Wilson ATPases. Using this complementation test, it was shown that mutation of the CPC motif to SPS in the Wilson ATPase, and to CAC or CPA in the *Caenorhabditis elegans* Menkes/Wilson homologue, resulted in non-complementing mutant ATPases [17,33,34]. These results agree with our data both *in vivo* and *in vitro* with the C396S CopB mutant.

The His-Pro dipeptide (HP) is another sequence motif specific to CPx-type ATPases, and its strict conservation suggests that it has a key role. It is located in the large cytoplasmic loop, about 40 amino acids downstream of the phosphorylation site with the signature Asp-Lys-Thr-Gly-Thr. In the Ca^{2+}-ATPase, this region is divided into two clearly separated domains, the phosphorylation domain (P), extending approximately eight amino acids beyond the Asp-Lys-Thr-Gly-Thr phosphorylation site, and the nucleotide-binding domain (N), formed by the remainder of the large cytoplasmic loop (cf. Figure 1). By analogy, the HP motif would be located in the N-domain near to the ATP-binding site, but there is no recognizable sequence similarity between the Ca^{2+}-ATPase and copper ATPases in the region of the HP motif.

The HP motif is affected by the most frequent Wilson disease mutation, H1069Q. The impact of the H1069Q mutation on Wilson ATPase function has been tested previously by functional complementation, but the findings remained contradictory. When expressed in fibroblasts of the mottled mouse, a model for Menkes disease, the Wilson ATPase gene carrying the H1069Q mutation was not able to rescue the mottled phenotype, whereas a wild-type Wilson gene could. The mutant enzyme was mislocalized to the endoplasmic reticulum at normal growth temperatures, and was degraded more rapidly than wild-type Wilson ATPase [35]. In contrast with this, several groups have shown that H1069Q could rescue the iron-uptake-deficient phenotype of a yeast *Ccc2* knock-out strain [17,36]. It had been speculated that this was a result of an overexpression of the mutated, yet slightly active, protein [17]. Because the level of expression was determined with antibodies on Western blots, it is inherently not possible to compare expression of complementing ATPase with normal expression of the endogenous Ccc2 copper ATPase in this system.

In the *E. hirae* system, we can compare expression levels of mutant ATPases with those of wild-type CopB directly. We here showed that H480Q CopB, corresponding to the H1069Q Wilson mutation, did not complement a CopB knock-out strain. Expression of the mutant enzyme was comparable with wild-type CopB levels. A promoter that is freely inducible and not affected by copper is not available for *E. hirae*, and therefore the effect of more extensive overexpression could not be tested. However, when non-functional copper ATPase is strongly overexpressed, copper resistance might arise by stoichiometric copper binding rather than transport out of the cell. This type of copper scavenging is most likely to be responsible for the slightly increased growth yields (shown by the final attenuance values) that were observed consistently for all strains expressing defective CopB, compared with cells not expressing any CopB protein. *In vitro*, H480Q CopB exhibited residual ATPase activity. Owing to the low activity of the enzyme, the kinetic parameters could not accurately be assessed.

Acylphosphate formation is a powerful tool to resolve partial reactions of ATPases in relation to the interaction with metal ions, and it has been used in the study of P-type ATPases [37,38]. As mentioned above, the function of CopB does not depend on added copper because of the presence of sufficient amounts of contaminating copper [19]. This does not permit the use of either ATP hydrolysis or acylphosphate formation to probe stimulation by metal ions. Nevertheless, the fact that we clearly observed phosphorylation and dephosphorylation in both mutants indicates that the mutations do not directly affect the ATP-binding site or the site of phosphorylation.

A point of concern in site-directed-mutagenesis experiments is the proper folding of mutant proteins. In the absence of an X-ray structure, which is not available for any copper ATPase, it is not possible to establish firmly whether mutant proteins are correctly folded. However, misfolding of the mutant ATPases is very unlikely, for the following reasons. First, purified mutant ATPases isolated from membranes were studied. These membranes contained similar amounts of wild-type and mutant ATPases, were extracted by dodecyl β-D-maltoside with the same efficiency as wild-type CopB, and displayed elution profiles identical with that of the wild-type in Ni-NTA–agarose and anion-exchange chromatography. Secondly, it has been observed, in a large number of site-directed mutagenesis studies with proteins of known X-ray structure, that the replacement of an amino acid by a residue of similar polarity and occupied volume does not lead to misfolding [39].

Taken together, here we have shown that the CPx and HP sequence motifs that have been conserved in all heavy-metal ATPases are key elements for enzyme function. Mutations in these motifs abolish copper transport, but not acylphosphate formation. Therefore the function of these motifs does not appear to be in the energy-providing step of the reaction cycle, but in a step accessory to, or linked with, it.

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