

Replacement of a cytosolic copper/zinc superoxide dismutase by a novel cytosolic manganese superoxide dismutase in crustaceans that use copper (haemocyanin) for oxygen transport

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The blue crab, *Callinectes sapidus*, which uses the copper-dependent protein haemocyanin for oxygen transport, lacks the ubiquitous cytosolic copper-dependent enzyme copper/zinc superoxide dismutase (Cu,ZnSOD) as evidenced by undetectable levels of Cu,ZnSOD activity, protein and mRNA in the hepatopancreas (the site of haemocyanin synthesis) and gills. Instead, the crab has an unusual cytosolic manganese SOD (cytMnSOD), which is retained in the cytosol, because it lacks a mitochondrial transit peptide. A second familiar MnSOD is present in the mitochondria (mtMnSOD). This unique phenomenon occurs in all Crustacea that use haemocyanin for oxygen transport. Molecular phylogeny analysis suggests the MnSOD gene duplication is as old as the origin of the arthropod phylum. cytMnSOD activity in the hepatopancreas changes during the moulting cycle of the crab. Activity is high in intermoult crabs and non-detectable in postmoult papershell crabs. mtMnSOD is present in all stages of the moulting cycle. Despite the

lack of cytCu,ZnSOD, crabs have an extracellular Cu,ZnSOD (ecCu,ZnSOD) that is produced by haemocytes, and is part of a large, approx. 160 kDa, covalently-linked protein complex. ecCu,ZnSOD is absent from the hepatopancreas of intermoult crabs, but appears in this tissue at premoult. However, no ecCu,ZnSOD mRNA can be detected, suggesting that the protein is recruited from the haemolymph. Screening of different taxa of the arthropod phylum for Cu,ZnSOD activity shows that those crustaceans that use haemoglobin for oxygen transport have retained cytCu,ZnSOD. It appears, therefore, that the replacement of cytCu,ZnSOD with cytMnSOD is part of an adaptive response to the dynamic, haemocyanin-linked, fluctuations in copper metabolism that occur during the moulting cycle of the crab.

Key words: copper metabolism, evolution, haemocyanin, moulting cycle, superoxide dismutase (SOD).

INTRODUCTION

The blue crab, *Callinectes sapidus*, is dependent, as with all other decapod crustaceans, on the copper protein haemocyanin for oxygen transport. Prior to moult, crabs stop feeding and catabolize haemocyanin, presumably as an energy reserve, resulting in release of potentially toxic copper [1]. At moult, crabs take up large amounts of water to provide the hydrostatic pressure needed to break open the old exoskeleton and to create increased living space. After moult, they have to restore the reduced haemocyanin concentrations to normal premoult levels, which requires a large amount of copper to be delivered to the secretory pathway for activation of haemocyanin, which is synthesized in the hepatopancreas. In view of these dynamic changes in copper metabolism during the animal's moulting cycle, blue crabs are attractive models in which to examine how animals detoxify excess toxic copper and make the metal available for the biosynthesis of copper-dependent proteins. Recent studies in our laboratory [1–3] have led to the discovery of novel forms of metallothionein (MT) and superoxide dismutase (SOD) in blue crabs that appear to be part of an adaptive strategy to enable them to cope with these large fluctuations in copper levels.

MTs constitute a family of low-molecular-mass cysteine-rich metal-binding proteins, which are found in microorganisms,

plants and all invertebrate and vertebrate animals. Blue crabs have two 'classical' Cd-MT isoforms, as found in vertebrates [4]. However, in contrast with vertebrates, crabs have a third copper-specific MT, denoted as CuMT3, which can be induced only by copper [3]. Levels of CuMT3 mRNA are highly correlated with changes in haemocyanin mRNA that occur during the crab's moulting cycle, suggesting that this copper-specific MT plays an important role in copper homeostasis associated with haemocyanin metabolism [1].

SODs are essential antioxidant enzymes that occur in virtually all oxygen-respiring organisms. They are classified into three distinct groups depending on the metal content: iron SOD (FeSOD) found in prokaryotes and in plants, manganese SOD (MnSOD) found in prokaryotes and in the mitochondria of eukaryotes, and copper/zinc SOD (Cu,ZnSOD) found in the cytosol and extracellular compartments of eukaryotes and in the periplasm of Gram-negative bacteria [5]. MnSODs from higher organisms are nuclear encoded mitochondrial matrix enzymes composed of four identical subunits of approx. 22 kDa. A larger precursor form of the enzyme is made in the cytosol and transported into the mitochondria, with subsequent cleavage of an N-terminal mitochondrial transit peptide [6]. The amino acid sequences of all MnSODs, whether from animals, plants or bacteria, are extremely similar and unrelated to those of the Cu,ZnSODs [5,7]

Abbreviations used: DTPA, diethylenetriaminepenta-acetic acid; MT, metallothionein; SOD, superoxide dismutase; Cu,ZnSOD, copper/zinc SOD; cytCu,ZnSOD, cytosolic Cu,ZnSOD; ecCu,ZnSOD, extracellular Cu,ZnSOD; MnSOD, manganese SOD; mtMnSOD, mitochondrial MnSOD; cytMnSOD, cytosolic MnSOD; RACE, rapid amplification of cDNA ends; UAP, universal amplification primer; UDG, uracil DNA glycosylase; UTR, untranslated region.

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The nucleotide sequence data reported for the blue crab cytMnSOD, mtMnSOD and ecCu,ZnSOD, brown shrimp cytMnSOD and mtMnSOD, and grass shrimp cytMnSOD will appear in DDBJ, EMBL, GenBank[®] and GSDB Nucleotide Sequence Databases under the accession numbers AF264030, AF264029, AF264031, AY211085, AY211086 and AY211084 respectively.

Cytosolic Cu,ZnSODs (cytCu,ZnSODs) are composed of two identical 16 kDa subunits. They are constitutively expressed and are considered to be essential 'house-keeping' enzymes [8]. The three-dimensional structures of bovine and human Cu,ZnSOD are highly conserved [9]. Amino acid sequences of cytCu,ZnSODs ranging from fungi to mammals are all very similar [5,7]. The virtually universal occurrence of cytCu,ZnSOD in eukaryotes and the highly conserved structure of the protein stress the importance of the enzyme in cellular homeostasis.

Vertebrates also have an extracellular Cu,ZnSOD (ecCu,ZnSOD) composed of four 22 kDa subunits [10]. The human ecCu,ZnSOD sequence in the region His⁹⁶ to Gly¹⁹³ shows strong homology with the cytCu,ZnSOD family, including the ligands for copper and zinc [10]. The ecCu,ZnSODs have evolved from the ancestral Cu,ZnSODs, before the differentiation of fungi, plants and metazoa, 1000 million years ago, indicating that its existence should be ubiquitous among eukaryotes [10,11].

Surprisingly, blue crabs, shrimps and lobsters lack cytCu,ZnSOD, but have a cytosolic MnSOD (cytMnSOD) instead [2]. In the present study, we report the complete cDNA and amino acid sequences of cytMnSOD and mitochondrial MnSOD (mtMnSOD) from the blue crab (*C. sapidus*), together with partial sequences of brown shrimp, *Farfantepenaeus aztecus*, and grass shrimp, *Palaemonetes pugio*, MnSODs. In addition, we demonstrate that crabs have an ecCu,ZnSOD, which is present in the haemolymph and produced by haemocytes, and show that the levels of cytMnSOD and ecCu,ZnSOD in the hepatopancreas change during the crab's moulting cycle. We present evidence indicating that these phenomena are linked to the use of copper for oxygen transport.

MATERIALS AND METHODS

Cloning of cytMnSOD

Excised hepatopancreas tissue from *C. sapidus* was frozen in liquid nitrogen and ground to a fine powder with a pestle and mortar. Total RNA was isolated from the powder using Tri Reagent (Sigma) and reverse-transcribed by 3'-rapid amplification of cDNA ends (3'-RACE) according to the protocol of the 3'-RACE System (Life Technologies). First-strand cDNA was then amplified utilizing a degenerate gene-specific primer GSP-1F (5'-AAYCCNCARGTNGCNGCNATG-3'), designed from the N-terminal amino acid sequence of blue crab cytMnSOD Asn-Pro-Gln-Val-Ala-Ala-Met [2], as the forward primer and the universal amplification primer (UAP; Life Technologies) as the reverse primer. A second nested amplification reaction was carried out with GSP-2F (5'-CAYATHAARGCNGARYTNGG-3'), designed from Hist-Ile-Lys-Ala-Glu-Leu-Gly [2], as the forward primer and UAP as the reverse primer. The nested GSP-2 was synthesized with CAUCAUCAUCAU attached to the 5'-end to facilitate uracil DNA glycosylase (UDG)-mediated cloning. An 825 bp amplification product was gel-purified using the QIAquick Gel Extraction kit (Qiagen) and UDG-cloned using the Clone Amp[®] pAMP1 System (Life Technologies). Plasmid DNA from several clones was isolated using the Wizard[®] Plus Miniprep DNA Purification System (Promega) and screened for inserts by digestion with *SalI* and agarose-gel electrophoresis. The 825 bp insert was sequenced by the University of Maine DNA Sequencing Center using the Sanger method [12]. A BLAST search [13] identified the sequence as that of MnSOD.

The 5'-end of the cDNA sequence was obtained by 5'-RACE according to the protocol of the 5'-RACE System (Life Technologies). Three complementary reverse gene-specific

primers, GSP-1R, GSP-2R and GSP-3R, were designed from the 3'-RACE sequence. First-strand cDNA was synthesized using the GSP-1R (5'-CAGCAAGCTTGTTCATCCTTAGGG-3') as the primer. The cDNA, after addition of a homopolymeric dC tail, was then amplified by PCR using GSP-2R (5'-GCCAGATCCTTTCACAGCAACAC-3') and the abridged anchor primer provided with the kit. Next, the PCR product was amplified with GSP-3R (5'-CAUCAUCAUCAUCCTGTTGATCATCTCAGCAATGG-3') as the reverse primer and UAP as the forward primer. The single product of approx. 725 bp was gel-purified, UDG-cloned and sequenced as described for the 3'-RACE product.

cytMnSOD from the brown shrimp and the grass shrimp was cloned using hepatopancreas cDNA as the template and the following primer pairs for the primary and nested PCR respectively: GSP-1F (5'-AAGGAWSYWTACATTKC-3') and GSP-1R (5'-GGRGCCATGTTDGTCCAGAARAT-3'); and GSP-2F (5'-GAGAAGAAGYTRGCTGAGCT-3') and GSP-2R (5'-TGRC-CMCCDCCATTGAACTTRAT-3'). The primer sequences were based on the alignment of the blue crab cytMnSOD sequence with that of the MnSOD of the tiger shrimp (*Penaeus monodon*), which had been deposited in GenBank[®] (accession number BI784454) during the course of the present study and appeared to correspond to the cytMnSOD form (see Figure 2). The gel-purified amplification product was cloned using the Promega pGEM[®]-T Easy Vector System and JM 109 competent cells (Promega A1360) and sequenced.

Cloning of mtMnSOD

Total RNA and cDNA were prepared as described above. The cDNA was amplified using GSP-1F (5'-GAYTAYGGC-GCNYTNGARCC-3') as the forward and GSP-1R (5'-ARRTARTANGCRTCCCA-3') as the reverse primer. The primers were derived from conserved amino acid sequences in the N- and C-terminal ends of MnSODs (see Table 1 in [11]). Two products approx. 575 bp and 475 bp long resulted. Both products were gel-purified and amplified with GSP-2F (5'-ACNATHWSNGC-NGARATHATG-3') designed from the N-terminal sequence of the mtMnSOD, Thr-Ile-Ser-Ala-Glu-Trp-Met [2], and GSP-1R. One amplification product of approx. 475 bp resulted. The DNA was gel-purified, cloned using the Promega pGEM[®]-T Easy Vector System (Promega A1360) and sequenced as described above. A BLAST search identified the sequence as MnSOD.

3'-RACE amplification was performed with GSP-3F, (5'-CCA-AGCACCATCAAACCTACGT-3', derived from the 475 bp nucleotide sequence) and abridged UAP provided with the 3'-RACE kit. Two amplification products of approx. 900 bp and 1200 bp were gel-purified, cloned in pGEM[®]-T Easy and sequenced. A BLAST search identified the 900 bp product as MnSOD.

Three gene-specific reverse primers, GSP-2R (5'-CTCA-ACACTGCCAAAATCACGA-3'), GSP-3R (5'-CTCAACACTGCCAAAATCACGA-3'), and GSP-4R (5'-TTATGTGTCCACC-ACCATTGA-3'), based on the 475 bp nucleotide sequence, were designed for 5'-RACE. The 5'-RACE protocol was the same as described for cytMnSOD. One amplification product of approx. 380 bp resulted. The DNA was gel-purified, cloned into pGEM[®]-T Easy and sequenced. A BLAST search identified the 380 bp product as MnSOD.

mtMnSOD from the brown shrimp, *F. aztecus*, was cloned using the following primer pairs for the primary and nested PCR respectively: GSP-1F (5'-AGATCATGVAVCTGCACCA-3') and GSP-1R (5'-TAGTAVGCRTGCTCCCA-3'); and GSP-2F (5'-AGGCACCACSMRRCCTACG-3') and GSP-2R (5'-CCNARC-CADCCCCAGCC-3'). The primers were based on the sequence

alignment of the blue crab mtMnSOD sequence with MnSOD sequences from the crab, *Charybdis feriatius*, the fruit fly (*Drosophila melanogaster*) and the tobacco hawkmoth (*Manduca sexta*) (see Figure 2 for accession numbers).

Cloning of ecCu,ZnSOD

Blue crabs contain an ecCu,ZnSOD present in the haemolymph and associated with the haemocytes (see below). For preparation of haemocytes, the soft tissue between an appendage and the ventral body of the crab was cleaned with 70% ethanol and punctured with a sterile 16-gauge needle. The haemolymph was collected directly in a solution of sterile seawater containing 2% caffeine and centrifuged for 10 min at 740 g to collect haemocytes. The resulting pellet was used for preparation of total RNA and first-strand cDNA as described above. Two forward primers GSP-1F (5'-GGNGGNCAYTTYAAYCC-3', corresponding to Gly-Gly-His-Phe-Asn-Pro) and GSP-2F (5'-CAYGCNGGNGAYTTYGGNAA-3', corresponding to His-Ala-Gly-Asp-Phe-Gly-Asn), and two reverse primers GSP-1R (5'-GCNCCNGC-RTTNCCNGT-3', corresponding to Thr-Gly-Asn-Ala-Gly-Ala) and GSP-2R (5'-TCRTCACNCCNGCRTCRTG-3', corresponding to His-Ala-Gly-Val-Asp-Asp) were designed for amplification of an internal sequence of ecCu,ZnSOD. The sequences of the primers were selected based on amino acid sequences that are conserved in both ecCu,ZnSODs and cytCu,ZnSODs (see Figures 4 and 5 in [11] and [14] respectively). cDNA was amplified using GSP-1F and GSP-1R, followed by amplification of the PCR products with GSP-2F and GSP-2R. One prominent amplification product of approx. 200 bp resulted. The 200 bp band was gel-purified, cloned into pGEM[®]T-Easy and sequenced as described above. A BLAST search identified the sequence as ecCu,ZnSOD.

Gene-specific forward primers GSP-3F (5'-CATGCTCGTG-ACTTCGGGAA-3') and GSP-4F (5'-CATTCAGCTGACCA-ATACG-3') for 3'-RACE were designed based on the 200 bp nucleotide sequence. cDNA was amplified using GSP-3F and abridged UAP, followed by amplification of the PCR products with GSP-4F and abridged UAP. Two prominent amplification products of approx. 280 bp and 550 bp resulted. The two DNA bands were gel-purified, cloned and sequenced. A BLAST search identified both products as ecCu,ZnSOD.

Three antisense primers were designed for 5'-RACE, GSP-3R (5'-CGTTTCCGGTCTTCAA-3'), GSP-4R (5'-CTATCAGCA-TTACCTCCGCG-3') and GSP-5R (5'-TCCCAGATCATCCTG-TCCTT-3'). The protocol was the same as described for cytMnSOD. Several amplification products were observed, with two prominent products of approx. 520 bp and 650 bp. The two DNA bands were excised, gel-purified and cloned. Three clones with inserts of different length (approx. 350, 425, 625 bp) were sequenced. A BLAST search identified all inserts as ecCu,ZnSOD.

Measurement of ecCu,ZnSOD mRNA

Haemocyte and hepatopancreas cDNA was used as a template for PCR amplification of ecCu,ZnSOD using TGTTCTCGG-TTCTCTAGCG (bp 41–61) as the forward primer and CCAG-ATCATCCTGTCTTGG (bp 598–618) as the reverse primer.

Purification of ecCu,ZnSOD

Haemolymph was collected from male blue crabs. Clotted haemolymph was homogenized and centrifuged for 10 min at

26 920 g. PMSF (0.1 mM) was added to the supernatant (addition of PMSF to haemolymph interferes with clotting), followed by centrifugation for 95 min at 416 000 g (Beckman Optima TL 100 Ultracentrifuge) to pellet haemocyanin. Supernatant was concentrated on a YM-10 Amicon ultrafiltration membrane, dialysed against 2 × 1 litre of 50 mM Tris/HCl (pH 7.6) (pH 8.2 in the cold), and centrifuged for 10 min at 11 960 g to remove precipitated material. Clarified supernatant was applied on to DEAE Cellulose (2.5 cm × 21 cm) in 50 mM Tris/HCl (pH 7.6) (pH 8.2 in the cold). The column was developed at a flow rate of 35 ml/h with a NaCl gradient generated from 500 ml of 50 mM Tris/HCl (pH 7.6) and 500 ml 50 mM Tris/HCl (pH 7.6) containing 1 M NaCl. SOD, identified by SDS/PAGE and SOD activity staining [15], eluted from the column between 240 and 335 mM NaCl. SOD-positive fractions were concentrated and applied on to Superdex HiLoad 16/60 (Amersham Biosciences) and developed with 50 mM Tris/HCl (pH 7.6) containing 0.1 mM diethylenetriaminepenta-acetic acid (DTPA) at a flow rate of 0.5 ml/min. Fractions of 2 ml were collected and SOD activity was found in fractions 23–26. Fractions were pooled, concentrated and applied on to a Sephacryl S300 16/60 (Amersham Biosciences) in 50 mM Tris/HCl (pH 7.6) containing 0.1 mM DTPA. Fractions of 2 ml were collected at a flow rate of 0.5 ml/min. SOD activity was found in fractions 25–30. Coomassie Blue staining of the SOD activity gels showed fractions 27–30 to be pure. To determine the molecular mass of SOD, the Sephacryl S300 16/60 column was calibrated with β -amylase (200 kDa), alcohol dehydrogenase (150 kDa), BSA dimer (132 kDa), BSA monomer (66 kDa) ovalbumin (43 kDa), carbonic anhydrase (29 kDa) and trypsin inhibitor (20.1 kDa). The molecular mass of denatured SOD was determined by SDS/PAGE on a 7.5% (w/v) polyacrylamide gel using Laemmli's buffer system [16]. The calibration line was composed of myosin (205 kDa), β -galactosidase (116 kDa), phosphorylase b (94 kDa), BSA (66 kDa), ovalbumin (43 kDa), and carbonic anhydrase (29 kDa). Protein bands were visualized with Coomassie Blue stain.

Screening of the arthropod phylogenetic tree for cytCu,ZnSOD

Blue crabs (*C. sapidus*), Gulf mud fiddler crabs (*Uca longisignalis*), striped-legged hermit crabs (*Clibanarius vittatus*), white shrimp (*L. setiferus*), grass shrimp (*P. pugio*) and mantis shrimp (*Squilla empusa*) were collected locally in the Mississippi Sound/Davis Bayou. Ghost crabs (*Ocypode quadrata*) and stone crabs (*Menippe mercenaria*) were collected in the vicinity of the Duke University Marine Laboratory, Beaufort, NC, U.S.A. Northern lobsters (*Homarus vulgaris*) were purchased locally. Freshwater crayfish (*Procambarus clarkii*) and millipedes were collected locally. All animals were in the intermolt stage. Hepatopancreas/midgut was removed from the animals and homogenized in 3 vols 50 mM potassium phosphate buffer (pH 7.8) containing 0.1 mM DTPA and 0.1 mM PMSF. Homogenates were centrifuged for 5 min at 16 000 g and the supernatants were passed through glasswool.

Amphipods (*Synurella*), aquatic Isopods (*Caecidotea*), terrestrial Isopods (*Armadilidium* and *Ligia*), mysid shrimp (*Mysidopsis almyra* and *Taphramysis louisiana*), barnacles (*Balanus improvegus*), copepods (*Acartia tonsa*) and insects (dragon fly larvae) were collected locally. Brine shrimp (*Artemia salina*), *Daphnia magna* and *Ceriodaphnia* were purchased from Ocean Star International. Krill (*Euphasia pacifica*) was a gift from Dr Marc Mangel (University of California at Santa Cruz, CA, U.S.A). The deep sea spider (*Anoplodactylus lentus*) was a gift from Harriet Perry (University of Southern Mississippi, Ocean



Figure 1 Amino acid sequence alignment of blue crab cytMnSOD and mtMnSOD with human mtMnSOD

Alignment was carried out with Clustal W 1.74 [17]. The sequence of cytMnSOD is that of the mature protein. The cDNA deduced amino acid sequence indicates that the sequence presented is preceded by MAEKDLYIAALEKKLAELSGIEVDQIKKNQLANASSEARSIREMAEYVEGIQVKQAGQVI (where single-letter amino acid notation is used). Amino acids in lower case indicate mitochondrial-targeting sequence. +, conserved amino acids responsible for binding manganese. *, lysine residues in human MnSOD involved in stabilizing the dimer-dimer interface. Grey shading refers to isofunctional amino acid residues.

Springs, MS, U.S.A.). All of the small animals were homogenized whole in 2 vols of 50 mM potassium phosphate buffer (pH 7.8) containing 0.1 mM DTPA and 0.1 mM PMSF. Tissue and whole-animal homogenates were subjected to PAGE and staining for SOD activity was carried out as described previously [15]. To distinguish between MnSOD and Cu,ZnSOD, gels were soaked in 5 mM KCN or 5 mM H₂O₂ to inhibit Cu,ZnSOD activity.

Changes of MnSOD and Cu,ZnSOD during the moulting cycle

Hepatopancreas tissue homogenates from crabs in different stages of the moulting cycle [1] were analysed for SOD activity as described above.

Western-blot analysis of cytCu,ZnSOD

Hepatopancreas tissues from blue crab, brown shrimp, grass shrimp and brine shrimp (*A. salina*) were homogenized in 2 vols of 50 mM Hepes (pH 7.5) containing 100 µM PMSF and 5 µg/ml aprotinin, and centrifuged at 16000 g for 5 min at 4 °C. Supernatants were diluted (1:1, v/v) with SDS-denaturation buffer, loaded on to 10% (w/v) polyacrylamide gels, and subjected to electrophoresis. Bovine cytCu,ZnSOD was used as a positive control. The gels were blotted electrophoretically on to PVDF membranes. cytCu,ZnSOD protein bands were detected using an anti-Cu,ZnSOD antibody (SOD-100; StressGen) in combination with the SuperSignal West Dura Western Blotting Kit for Rabbit IgG Detection (Pierce). Chemiluminescence was quantified using a Bio-Rad Fluor-S MultiImager.

Molecular phylogeny analysis

The amino acid sequences of blue crab cytMnSOD and mtMnSOD were aligned with sequences from 13 other species using Clustal W 1.74 [17]. The amino acid sequence of blue crab ecCu,ZnSOD was aligned with sequences from five ecCu,ZnSODs and eight cytCu,ZnSODs. The aligned sequences were inputted into the SEQBOOT algorithm from Felsenstein's PHYLIP package [18] to generate 100 data sets for MnSOD and ecCu,ZnSOD by bootstrap resampling [19]. The multiple data sets were used to calculate 100

most parsimonious trees with PROTPARS [18]. The resulting tree output file, composed of the 100 best trees found among the 100 runs, was used as input in the program CONSENSE that calculates a majority rule consensus tree with confidence intervals. The 100 aligned sequences produced by SEQBOOT were also used to calculate 100 protein distance matrices using PROTDIST [18]. Phenograms were then calculated with the matrices and the neighbour-joining method [20] using NEIGHBOR [18], and converted into a majority rule consensus tree with CONSENSE.

RESULTS

Cloning and characterization of cDNAs encoding the cytosolic and mitochondrial MnSOD

The complete 1176 bp cDNA sequence of the cytMnSOD of the blue crab was obtained from a 639 (1–639) bp 5'-RACE and a 867 (310–1176) bp 3'-RACE product. The cDNA has a 90 bp 5'-untranslated region (UTR), a 855 bp open reading frame encoding a 285 amino acid 31.4 kDa protein (Figure 1), and a 230 bp 3'-UTR, with a polyadenylation signal at position 1134. The protein purified from the cytosol starts at amino acid residue 60 [2], suggesting that residues 1–59 may constitute a leader sequence. However, this peptide does not have any of the properties of either import or export leader sequences [21]. The molecular mass of the mature protein is 24.8 kDa. The mature protein shows 60% identity with the mtMnSOD protein, including the ligands for manganese binding. The translated partial cDNA sequences of the cytMnSODs from the brown and grass shrimps cloned in the present study correspond to amino acids 12–154 of blue crab cytMnSOD and show 75 and 76% identity respectively, with this sequence. These two sequences are available from GenBank® under the accession numbers AY211085 and AY211084.

The 1147 bp cDNA of mtMnSOD was determined from three cloned PCR products. The first sequence was obtained from a PCR product amplified with forward and reverse primers derived from conserved amino acid sequences. This internal sequence (162–593) allowed for design of 3'- and 5'-RACE primers. The 5'-RACE product gave residues 1–639, and the 3'-RACE product residues 197–1147. The open reading frame (49–705) encodes a 218 amino acid protein, with nucleotides 49–114 encoding a

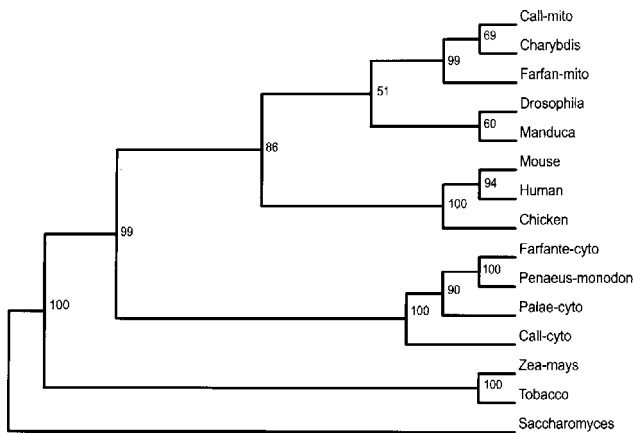


Figure 2 Phylogenetic relationships of plant, invertebrate and vertebrate MnSODs, including cytMnSOD and mtMnSOD from the blue crab

The phenogram was calculated with the neighbour-joining method as described in the Materials and methods section. *S. cerevisiae* MnSOD was used as the outgroup. The numbers at the forks indicate the number of times the group consisting of the species which are to the right of that fork occurred among the 100 calculated trees. Species and accession numbers from the National Center of Biotechnology Information Entrez Protein Database (unless otherwise specified) are: *Saccharomyces* (*S. cerevisiae*, CAA26092), Tobacco (*Nicotiana plumbaginifolia*, CAA32643), *Zea mays* (AAA72022), Call-cyto (blue crab cytMnSOD, AAF74771), Palae-cyto (grass shrimp cytMnSOD, GenBank® accession number AY211084), *Penaeus-monodon* (tiger shrimp, GenBank® BI784454), Farfante-cyto (brown shrimp cytMnSOD, AY211085), Chicken (*Gallus gallus*, AAG46055), Human (*Homo sapiens*, CAA68791), Mouse (*Mus musculus*, NP_038699), Manduca (*Manduca sexta*, GenBank® accession number BF047053), *Drosophila* (*D. melanogaster*, AAA20553), Farfan-mito (brown shrimp mtMnSOD, GenBank® accession number AY211086), *Charybdis* (*Ch. feriatus*, AAD10640) and Call-mito (blue crab mtMnSOD, AAF74770).

22 amino acid mitochondrial-targeting sequence (Figure 1). The molecular mass of the mature protein is 21.7 kDa. The 3'-UTR contains a polyadenylation signal at position 1122. The translated partial cDNA sequence of the mtMnSOD from the brown shrimp cloned in the present study (GenBank Acc. No. AY211086) corresponds to amino acids 51–146 of blue crab mtMnSOD and shows 84% identity with this sequence.

Molecular phylogeny analysis of MnSOD

Molecular phylogeny analysis (Figure 2) shows that the cytMnSOD sequence of the blue crab clusters firmly with cytMnSOD sequences of the brown and grass shrimps, which were cloned in the present study, and with the MnSOD sequence from the tiger shrimp (*Penaeus monodo*, GenBank® accession number BI784454), indicating that the tiger shrimp sequence is that of a cytMnSOD form. The mtMnSOD from the blue crab clusters with the mtMnSOD from the brown shrimp (cloned in the present study) and the MnSOD from the crab *Ch. feriatus* (GenBank® accession number AF019411). The occurrence of two forms of MnSOD present in two different cellular compartments thus appears common to crabs and shrimp (decapod crustaceans).

Purification and characterization of blue crab ecCu,ZnSOD

ecCu,ZnSOD was purified by a combination of ion-exchange and size-exclusion chromatography. SDS/PAGE of the purified native SOD showed a single protein band, which barely enters the gel (R_f approx 0.1), and which coincides with a cyanide-sensitive SOD-active band. The molecular mass of native SOD

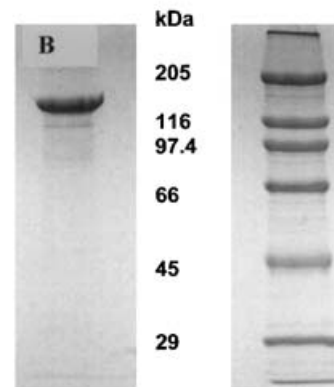
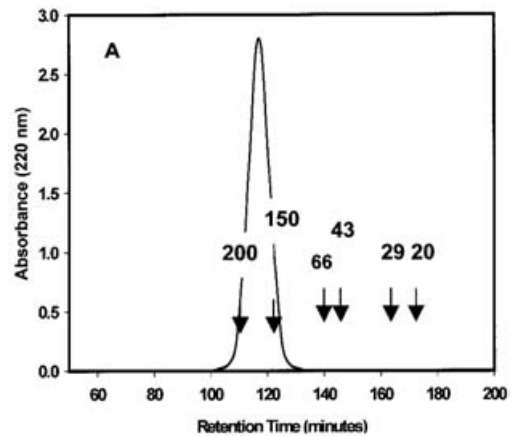


Figure 3 Sephacryl S300 elution profile (A) and SDS/PAGE gel (B) of purified ecCu,ZnSOD

Molecular-mass markers in (A) are expressed in kDa and are indicated by the arrows. The molecular mass of the ecCu,ZnSOD determined from the Sephacryl and SDS/PAGE calibration curves is 173 kDa and 146 kDa respectively.

determined by size-exclusion chromatography on Sephacryl S300 was approx. 173 kDa (Figure 3A). SDS/PAGE gels showed one protein band, with an estimated molecular mass of approx. 146 kDa (Figure 3B).

Monitoring of SOD activity and protein concentrations during the purification steps, using the cytochrome *c* reduction assay [22] and the bicinchoninic acid assay [23] respectively, revealed that approx. 85% of SOD activity was lost after centrifugation of the haemolymph, indicating that the majority of SOD does not occur in solution and suggesting that SOD might be associated with the cell surface of haemocytes. To explore this possibility, haemolymph and haemocytes were collected by centrifugation as described in the Materials and methods section. Supernatant and homogenized haemocytes were subjected to PAGE and gels were stained for SOD activity. Supernatants contained only the approx. 160 kDa cyanide-sensitive Cu,ZnSOD, whereas haemocytes contained both high-molecular-mass cyanide-sensitive Cu,ZnSOD and cyanide-insensitive MnSOD. The absence of MnSOD in the supernatants indicates that the haemocytes were intact. It appears therefore that the high-molecular-mass SOD in the haemolymph is released from the haemocyte surface. These results also indicate that blue crab haemocytes lack the 32 kDa cytCu,ZnSOD, since the only cyanide-sensitive SOD activity on the gel was associated with the approx. 160 kDa ecCu,ZnSOD protein. The haemocyte SOD was cloned and



Figure 4 Amino acid sequence alignment of the ecCu,ZnSODs from blue crab, crayfish and human

Alignment was carried out with Clustal W 1.74 [17]. Amino acids in lower case indicate signal sequence. +, amino acids responsible for binding copper; *, amino acids responsible for binding zinc. Crayfish ecCu,ZnSOD, GenBank[®] accession number AF122900; and human ecCu,ZnSOD, National Center of Biotechnology Information Entrez Protein Database accession number P08294.

sequenced, which allowed unequivocal identification of the SOD as an ecCu,ZnSOD, as discussed below.

Cloning and sequencing of the ecCu,ZnSOD

The complete 1035 bp nucleotide sequence of ecCu,ZnSOD (Figure 4) was obtained from cDNA prepared from haemocytes. The first PCR amplification product, obtained with primers derived from conserved Cu,ZnSOD amino acid sequences, consisted of a 157 bp product encoding 52 amino acids (residues 127–179; Figure 4).

3'-RACE, using primers derived from the 157 nucleotide sequence, gave two major amplification products. The first product (bp 486–767) was composed of a 204 bp coding sequence (amino acids 127–194) and a 77 bp 3'-UTR. The second (bp 486–1035) was the same as the first with an additional 269 bp at the 3'-end. The presence of the smaller 3'-RACE product is due to the occurrence of a polyadenylated sequence (757–770) in the 3'-UTR, which served as a binding site for the poly(dT) primer used for first strand cDNA reverse transcription. 5'-RACE gave three major products. The largest product consisted of 619 bp and contained the translation start at position 113. The smaller amplification products were truncated forms (196–619 and 281–619) of the 619 bp sequence. Based on alignment with the amino acid sequence of the ecCu,ZnSOD from the crayfish *Pacifastacus* [14], the blue crab ecCu,ZnSOD has a putative signal sequence composed of 36 amino acid residues (Figure 4). Primers derived from the region of the nucleotide sequence of ecCu,ZnSOD that contains the ligands for copper and zinc and is conserved in cytCu,ZnSODs failed to amplify hepatopancreas cDNA, indicating the lack of detectable levels of cytCu,ZnSOD mRNA in this tissue.

Molecular phylogeny analysis of ecCu,ZnSOD

Molecular phylogeny analysis shows the blue crab ecCu,ZnSOD clusters firmly with the ecCu,ZnSOD family (Figure 5). The ecCu,ZnSODs arose by gene duplication of an ancestral Cu,ZnSOD gene before the plant–animal divergence a billion

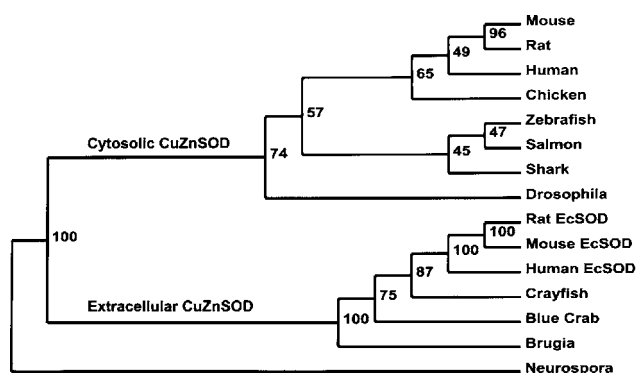


Figure 5 Phylogenetic relationships of cytCu,ZnSOD and ecCu,ZnSOD, including the haemocyte Cu,ZnSOD, from the blue crab

A most parsimonious tree was calculated as described in the Materials and methods. *Neurospora crassa* Cu,ZnSOD was used as the outgroup. The numbers at the forks indicate the number of times the group consisting of the species which are to the right of that fork occurred among the 100 calculated trees. Species and accession numbers from the National Center of Biotechnology Information Entrez Protein Database (unless otherwise specified) are: *Neurospora* (*N. crassa*, AAA63780), *Brugia* (*Brugia pahangi* ecCu,ZnSOD, CAA53901), Blue Crab (*C. sapidus*, AAF74772), Crayfish (*P. leniusculus*, AAD25400), Human ecCu,ZnSOD (*H. sapiens*, DSHUEC), Mouse ecCu,ZnSOD (*M. musculus*, NP_035565), Rat ecCu,ZnSOD (*Rattus norvegicus*, NP_037012), *Drosophila* (*D. melanogaster*, AAA28906), Shark (*Prionace glauca*, SO4623), Salmon (*Salmo salar*, GenBank[®] accession number BG936553), Zebrafish (*Danio rerio*, CAA72925), Chicken (*G. gallus*, AAB88059), Human (*H. sapiens*, CAA26182), Rat (*R. norvegicus*, P07632), Mouse (*M. musculus*, CAA29880).

years ago [11]. It appears therefore that crabs, despite the presence of the Cu,ZnSOD precursor gene, have developed a novel way of cytosolic antioxidant defence by using cytMnSOD instead of Cu,ZnSOD.

Screening of the arthropod phylogenetic tree for cytCu,ZnSOD and MnSOD activity

Representative organisms of different crustacean taxa were screened for the presence of cytCu,ZnSOD (Figure 6). All

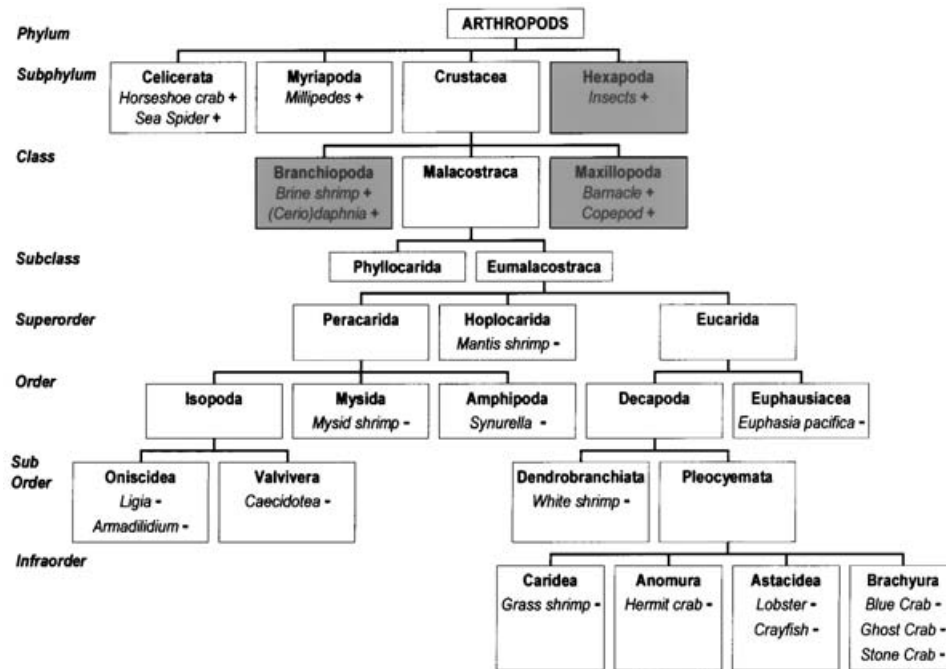


Figure 6 Depiction of the crustacean taxonomy

Species representative of different taxa were screened for the presence (+) or absence (–) of cytCu,ZnSOD using activity staining of polyacrylamide gels of tissue or whole-animal homogenates. All crustaceans dependent on copper (haemocyanin, open boxes) for oxygen transport (Malacostraca) have only cyanide-insensitive MnSOD. No cyanide-sensitive Cu,ZnSOD is observed. Crustaceans that use iron (haemoglobin, grey boxes) for oxygen transport (Branchiopoda and Maxillopoda) have cytCu,ZnSOD. Details of the taxonomy are given in [43].

Crustaceans that use copper (haemocyanin) for oxygen transport (class Malacostraca) have only cyanide-insensitive MnSOD. In contrast, crustaceans that use haemoglobin for oxygen transport such as barnacles, copepods (class Maxillopoda), brine shrimp and daphnia (class Branchiopoda) [24] do have a cyanide-sensitive Cu,ZnSOD, which has an R_F value of approx. 0.5, similar to that of bovine cytCu,ZnSOD. Western-blot analysis (see below) confirms that this SOD corresponds to cytCu,ZnSOD. It appears therefore that the replacement of Cu,ZnSOD in crustaceans with a cytosolic form of MnSOD is correlated with the high requirement for copper for haemocyanin synthesis. However, this scenario does not apply to Chelicerata (spiders and horseshoe crabs) and Myriapoda (millipedes) (Figure 6), which also use haemocyanin for oxygen transport, but have cytCu,ZnSOD. This issue will be further addressed in the Discussion.

Western-blot analysis of cytCu,ZnSOD

Western blots show that blue crabs, brown and grass shrimps have non-detectable levels of the cytCu,ZnSOD protein. In contrast, *Artemia*, a crustacean that does have cytCu,ZnSOD activity (Figure 6), does show a 16 kDa band that binds with the anti-Cu,ZnSOD antibody (Figure 7). It appears therefore that the lack of cytCu,ZnSOD activity in crabs and shrimps is due to the absence of cytCu,ZnSOD protein.

Changes of MnSOD and ecCu,ZnSOD during the moulting cycle

The data presented in Figure 8 show that the cytMnSOD, which is present as a diffuse band on PAGE gels due to the fact that the protein exists in monomer–dimer equilibrium [2], completely

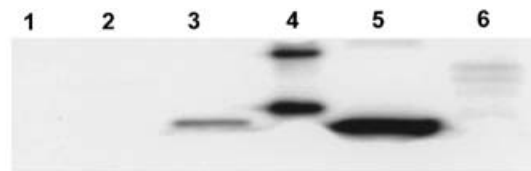


Figure 7 Western blot of Cu,ZnSOD

Lane 1, grass shrimp (150 μ g of hepatopancreas protein); lane 2, brown shrimp (230 μ g of hepatopancreas protein); lane 3, brine shrimp (10 μ g of whole-body protein); lane 4, molecular-mass markers (20 kDa and 30 kDa); lane 5, bovine Cu,ZnSOD (10 ng); lane 6, blue crab (85 μ g of hepatopancreas protein).

disappears postmoult during the papershell stage, whereas the mtMnSOD is present during all stages of the moulting cycle.

ecCu,ZnSOD is not present in the hepatopancreas of intermoult crabs, but appears in this tissue in the premoult and postmoult stages. The identity of the high-molecular-mass SOD band on the gel ($R_F = 0.1$) as ecCu,ZnSOD was confirmed by Sephacryl chromatography of hepatopancreas tissue homogenates of premoult crabs, which indicated a molecular mass of approx. 173 kDa. The changes in ecCu,ZnSOD and cytMnSOD are highly reproducible and have been confirmed for three independent sets of blue crabs. PCR amplification of cDNA from the hepatopancreas of 30 crabs in different stages of the moulting cycle using ecCu,ZnSOD-specific primers failed to show the presence of ecCu,ZnSOD mRNA. This indicates that the ecCu,ZnSOD is not synthesized in the hepatopancreas, but is probably recruited from the haemolymph where it is synthesized by the circulating haemocytes. The decrease in total SOD activity that accompanies the transition from intermoult to papershell crab

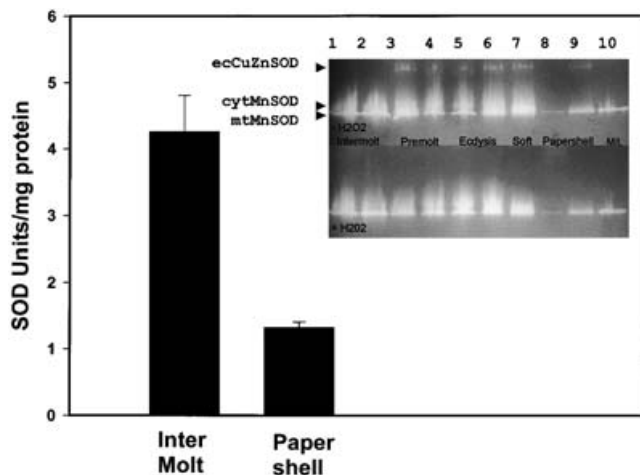


Figure 8 SOD activity in hepatopancreas tissues from crabs in different stages of the moulting cycle as determined by spectrophotometric solution assay and staining of polyacrylamide gels for SOD activity

Spectrophotometric solution assay of SOD activity at the intermolt and papershell stages is shown. Inset, two gels run in the absence (upper gel) or presence (lower gel) of H_2O_2 to inhibit Cu,ZnSOD activity. Lanes 1 and 2 contain tissue homogenates from intermolt crabs; lanes 3 and 4 from premolt crabs; lanes 5 and 6 from crabs just after ecdysis; lane 7 from soft crab; lanes 8 and 9 from papershell crabs; lane 10 is an extract from purified blue crab hepatopancreas mitochondria. The uppermost band in the upper gel, which disappears after H_2O_2 treatment as shown in the lower gel, is the 160 kDa ecCu,ZnSOD, as confirmed by Sephacryl chromatography of hepatopancreas homogenates of premolt crabs. The diffuse H_2O_2 -insensitive band in lanes 1–7 in the upper and lower gels is the cytMnSOD. The distinct H_2O_2 -insensitive band, moving with the front of the cytMnSOD in lanes 1–7 and as a separate band in lanes 7–10, is the mtMnSOD. The disappearance of the cytMnSOD in papershell crabs observed on the gel (lanes 8 and 9) is confirmed by the spectrophotometric assay for total SOD activity, which shows a significant decrease in SOD activity in papershell crabs ($n = 4$; error bar represents S.E.M.; $P < 0.005$).

was confirmed by a spectrophotometric solution assay for SOD activity, which showed 4.26 ± 0.55 SOD units/mg of protein in intermolt crabs and 1.32 ± 0.09 SOD units/mg of protein ($n = 4$; means \pm S.E.M.; $P < 0.005$) in papershell crabs (Figure 8).

DISCUSSION

cytCu,ZnSOD is a house-keeping enzyme that is essential for intracellular antioxidant defence and occurs in all eukaryotes, except, as we have shown in the present study, in crustaceans that use haemocyanin for oxygen transport. The catalytic function of the enzyme is the dismutation of the superoxide anion into O_2 and H_2O_2 . In yeast, the protein may also play a role in buffering of copper and in metabolism of zinc [25,26]. Cu,ZnSOD has been localized to the cytosolic cellular compartment, whereas MnSOD is localized to the mitochondria. However, recent studies [27,28] have shown that Cu,ZnSOD in both yeast and rats can also be found in the intermembrane space of mitochondria, whereas the studies reported in the present study show that distinct forms of MnSOD in crustaceans can be found in both mitochondria and cytosol.

Cloning and sequencing of the cytosolic and mitochondrial cyanide-insensitive SODs from the blue crab and from the brown and grass shrimps, firmly establishes their identity as belonging to the MnSOD family. The sequences of the mature cytMnSOD and mtMnSOD show only 60% identity, suggesting that the gene duplication giving rise to the two MnSODs is ancient. This is confirmed by phylogenetic analysis using neighbour-join-

ing and maximum parsimony methods. The phylogenetic tree, constructed from plant, invertebrate and vertebrate MnSOD sequences, indicates that the cytMnSOD–mtMnSOD gene duplication occurred after the plant–metazoa (animals) divergence (1000 million years ago) and predates the divergence of the protostomes (which include the arthropods) and deuterostomes (which include the chordate/vertebrates) [29], which took place approx. 540 million years ago [30]. It appears therefore that the MnSOD gene duplication is probably as old as the origin of the arthropod phylum more than 500 million years ago. Screening of arthropod species for SOD activity shows that the disappearance of cytCu,ZnSOD (and its replacement by a MnSOD) coincides with the divergence of the Crustacea and Chelicerata which, based on the fossil record and on molecular phylogeny analyses of their haemocyanin sequences, occurred in the Cambrium, some 600 million years ago [31,32], in good agreement with the results of the MnSOD molecular phylogeny analysis.

Comparison of the cDNA-derived amino acid sequence of the cytMnSOD with the N-terminal sequence of the purified protein [2] shows that cytMnSOD is synthesized as a precursor protein with a 59 amino acid peptide preceding the sequence of the mature protein. Western blotting of hepatopancreas cytosol using anti-MnSOD antibodies shows only the presence of the mature protein of approx. 25 kDa (results not shown), indicating that the N-terminal peptide is removed from the precursor protein of approx. 31 kDa immediately after translation. This peptide shows 75% identity with the N-terminal sequence of the MnSOD of the tiger shrimp (GenBank® accession number BI784454). The secondary structure of both peptides was calculated with PELE version 3.2 (Biology WorkBench <http://workbench.sdsc.edu>), which uses seven different algorithms to predict a consensus structure. Both the blue crab and shrimp peptides are predicted to consist of two α -helices (amino acids 3–19 and 23–47 for the blue crab and 3–19 and 23–54 for the shrimp) and a C-terminal random coil (amino acids 48–60 for the blue crab and 54–60 for the shrimp). Calculation of the secondary structure of the N-terminal peptides of the cytMnSODs from the brown and grass shrimps, which start at amino acid 12, predict an α -helix at residues 23–47 and a random coil at residues 54–60 for both species. The conservation of both primary and secondary structure of the N-terminal peptide of the four cytMnSODs suggests that the peptide has a defined biological function, which at present is unknown.

The mtMnSODs from blue crab and mammals are tetrameric proteins [33], whereas the cytMnSOD from the crab is a dimeric protein, which exists in a monomer–dimer equilibrium [2]. There are three lysine residues on the surface of helices in positions 75, 130 and 154 of the human enzyme (Figure 1) that form important contacts with other subunits at the dimer–dimer interface [33]. Alignment of the human and crab MnSOD sequences shows that the corresponding residues in the tetrameric crab mtMnSOD are lysine, asparagine and lysine, and in the dimeric cytosolic crab protein are alanine, serine and proline (Figure 1). The absence of the positively charged lysine residues may explain why the cytosolic form does not form tetramers.

The absence of cytCu,ZnSOD in the blue crab is supported by the following evidence. First, no cyanide-sensitive SOD activity can be detected in hepatopancreas and gills of intermolt crabs. A high-molecular-mass (approx. 160 kDa) cyanide-sensitive ecCu,ZnSOD is observed in the hepatopancreas of crabs that are in the process of moulting. This protein can be easily distinguished from the 32 kDa cytCu,ZnSOD, which is present in haemoglobin-containing crustaceans, by its high-molecular mass and low R_F (approx. 0.1) value on polyacrylamide gels. Secondly, even after copper exposure of animals or hepatopancreas tissue explants, which results in high levels of copper in the hepatopancreas, no

cyanide-sensitive Cu,ZnSOD is observed [34]. Thirdly, treatment of tissue homogenates with Cu⁺-glutathione, which is capable of restoring the enzymic activity of apoCu,ZnSOD [35] does not lead to measurable levels of Cu,ZnSOD activity (M. Brouwer, unpublished results), indicating that no apoCu,ZnSOD is present. Fourthly, no cytCu,ZnSOD protein is observed by Western-blot analysis using an anti-Cu,ZnSOD antibody that binds with cytCu,ZnSOD from haemoglobin-containing crustaceans. Finally, primers designed from the region of the nucleotide sequence of ecCu,ZnSOD, which contains the ligands for copper and zinc and is conserved in cytCu,ZnSODs, fail to amplify hepatopancreas cDNA, indicating non-detectable levels of cytCu,ZnSOD mRNA in this tissue. Although proving a negative is always difficult, taken together, the evidence presented above constitutes a compelling argument in support of the conclusion that, in the blue crab, other crabs and shrimps, the cytMnSOD has replaced cytCu,ZnSOD.

Despite the lack of a cytCu,ZnSOD, the blue crab has an ecCu,ZnSOD that is produced by haemocytes. The calculated mass of the protein, based on the cDNA-derived amino acid sequence, is 20.3 kDa. However, the measured mass of the native and SDS-denatured protein is approx. 160 kDa. The presence of the reducing agent mercaptoethanol in the SDS-denaturation buffer did not reduce the molecular mass, indicating that the 160 kDa complex is not held together by disulphide bridges. It appears likely, therefore, that the ecCu,ZnSOD is part of a larger covalently linked protein complex as found for crayfish ecCu,ZnSOD, which is covalently linked to peroxinectin, the homologue of human myeloperoxidase [14]. However, the possibility that the molecular mass discrepancy is due to carbohydrate binding to ecCu,ZnSOD cannot be excluded. Using gene-specific primers, we were unable to detect ecCu,ZnSOD mRNA in blue crab hepatopancreas. It appears that the crab has lost the gene that encodes cytCu,ZnSOD. The gene encoding ecCu,ZnSOD is still active and is expressed in a functionally specific blood cell involved in immune defence.

What possible advantage made MnSOD successful over Cu,ZnSOD in the cytosolic compartment? We hypothesized that the lack of cytCu,ZnSOD might be correlated with the high requirement for copper for synthesis of haemocyanin. Haemocyanins have been found in all euarthropod subphyla, possibly derived from a phenoloxidase-like enzyme. Thus haemocyanins were present in the last common ancestor of the Chelicerata, Crustacea, Myriapoda and Hexapoda [32]. To test our hypothesis, we screened species from the four subphyla for the absence or presence of cytCu,ZnSOD. As shown in Figure 6, we found that, in the Chelicerata and Myriapoda, haemocyanin and cytCu,ZnSOD co-exist. However, in the Crustacea the co-existence of haemocyanin and cytCu,ZnSOD appears to have become incompatible. In Branchiopoda and Maxillopoda, this incompatibility has been solved by replacing haemocyanin with an echaemoglobin, while retaining cytCu,ZnSOD. In the Malacostraca, which represent 20000 described living species, cytCu,ZnSOD has been replaced with MnSOD while retaining haemocyanin. Why can haemocyanin and Cu,ZnSOD coexist in the Chelicerata? In this group of organisms, haemocyanin is synthesized in highly specialized cells (cyanoblasts), located in the inner heart wall (spiders) or in tissue behind the compound eye (horseshoe crab) [36–38]. The chelicerate haemocyanins lack a signal peptide to direct the protein to the secretory pathway [39]. This is consistent with the observation that in chelicerates haemocyanin is synthesized by free ribosomes, accumulates to very high crystalline-like concentrations and is released by cell rupture or holocrine secretion [38]. In contrast, crustaceans, including the blue crab, synthesize haemocyanin with a signal peptide in hepatopancreas cells, which

also synthesize SODs [1]. In summary, it appears that arthropods have evolved three distinct strategies to avoid the potential 'intracellular apohaemocyanin, apoSOD, copper' conflict: (i) by synthesizing haemocyanin and cytCu,ZnSOD in separate tissues (Chelicerata); (ii) by replacing haemocyanin with haemoglobin (Branchiopoda and Maxillopoda), and (iii) by replacing a copper-dependent SOD with a manganese-dependent enzyme (Malacostraca). How the Myriapoda fit in within this scheme is unclear, since we do not know where haemocyanin is synthesized in these organisms.

The medfly, *Ceratitis capitata*, has two genes *ccSOD1* and *ccSOD2* encoding different forms of Cu,ZnSOD [40]. *ccSOD1* is expressed mainly in the early embryo and *ccSOD2* in the adult fly, but not in the early developmental stages. To examine if a cytCu,ZnSOD might be expressed during different stages of the crab's moulting cycle, we analysed hepatopancreas tissues from intermoult, premoult, ecdysis, softshell and papershell crabs for SOD activity. No 32 kDa cytCu,ZnSOD was observed in any of the moulting cycle stages. Surprisingly, the 160 kDa ecCu,ZnSOD was present in premoult, ecdysis, softshell and papershell crabs, but not in intermoult crabs. The absence of ecCu,ZnSOD mRNA suggests that the protein was not synthesized in the hepatopancreas, but recruited from the haemolymph. The appearance of the ecCu,ZnSOD in the premoult crabs coincides with the degradation of haemocyanin and concomitant release of copper, which occurs in the hepatopancreas of premoult, ecdysis and softshell crabs [1]. The ecCu,ZnSOD may function to protect the cell membrane from oxidative damage by preventing the copper-catalysed Fenton reaction $O_2 + H_2O_2 \rightarrow O_2 + OH^\bullet + OH^-$, with the increased flux of O_2 due to reaction of Cu(II) with GSH to give Cu(I) and the thiyl radical, GS^\bullet . GS^\bullet reacts with the thiolate anion GS^- to give $GSSG^{\bullet-}$. The latter is a strongly reducing species that reacts rapidly with O_2 : $GSSG^{\bullet-} + O_2 \rightarrow O_2^- + GSSG$ [34,41].

mtMnSOD is present in all stages of the moulting cycle. cytMnSOD disappears postmoult during the papershell stage, which is characterized by the resumption of synthesis of haemocyanin needed to replenish the depleted haemocyanin stores [1]. We also observed that the activity of the antioxidant enzyme glutathione peroxidase, which is up-regulated in blue crabs in response to copper exposure [34], decreased dramatically during the paper shell stage: 5.14 ± 1.28 units/mg of protein in intermoult crabs and 0.57 ± 0.36 units/mg of protein in papershell crabs ($n = 4$; means \pm S.E.M.; $P < 0.031$; M. Brouwer and T. Hoexum Brouwer, unpublished results). These decreases in antioxidant enzyme activities suggest that the copper-induced oxidative stress experienced by premoult, ecdysis and softshell crabs is alleviated in papershell crabs, which actively remove copper from the hepatopancreas in the form of haemocyanin. Even though the interpretation of the observed results is speculative, the observed changes in antioxidant enzyme activities underscore further the usefulness of the blue crab as a model for the study of the dynamics of copper metabolism and related defence mechanisms.

The present study and earlier work in our laboratory demonstrate that crabs and other decapod crustaceans that are dependent on copper (haemocyanin) for oxygen transport have developed unique features of copper metabolism. Whereas most animals, and aerobic eukaryotes in general, have a cytosolic copper-dependent form of SOD, crabs have a manganese-dependent enzyme. Vertebrate animals and crabs have at least two cadmium-inducible forms of MT, but crabs have a third, unique MT (CuMT3) that can only be induced by copper [1,3]. Levels of CuMT3 mRNA are positively correlated to levels of haemocyanin mRNA during the crab's moulting cycle [1]. Whereas most organisms use an

ATP-driven copper pump to move copper from the cytosol to the endoplasmic reticulum/Golgi network [42], carrier-mediated copper uptake by crab microsomes does not require ATP and is not affected by the P-type Cu-ATPase inhibitor orthovanadate [1]. Current studies in our laboratory are aimed at elucidating the mechanisms of transcriptional control of the MnSOD isoforms, the mechanism of ecCu,ZnSOD recruitment and identification of the transcription factor(s) that control the coordinate expression of CuMT3 and haemocyanin during the moulting cycle.

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