Unexpected expression of carbonic anhydrase I and selenium-binding protein as the only major non-heme proteins in erythrocytes of the subterranean mole rat (*Spalax ehrenbergi*)

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Abstract Chromatographic separation of the non-heme proteins from the erythrocytes of the subterranean mole rat belonging to the superspecies *Spalax ehrenbergi* from Israel revealed two major peaks. On sequence analyses, the larger peak corresponded to a 56 kDa selenium-binding protein (SeBP) previously characterized from mouse and human liver, and the second peak to the low-activity carbonic anhydrase (CA) isozyme, CA I. There was no evidence of the high-activity CA II isozyme normally found in the red cells of all amniotes tested to date. Thus, the mole rat appears to be the first mammalian species to express both a SeBP and the low-activity CA I isozyme, as the major non-heme proteins in its red blood cells. It is possible that the absence of the high-activity CA II isozyme may be advantageous to the mole rat in adapting to the low O_2 and high CO_2 environment of its underground burrows. It is also likely that the 56 kDa SeBP may play an important adaptive role in the physiology of the red cell.

Key words: Carbonic anhydrases I and II; Selenium-binding protein; Hypoxia; Hypercapnia; Respiratory acidosis; Mole rat erythrocyte

1. Introduction

The subterranean, blind mole rat superspecies *Spalax ehrenbergi* (family Spalacidae) from the eastern Mediterranean is a rodent that maintains high metabolic rates in adapting to the low oxygen (6–15%) and high carbon dioxide (5–13%) atmosphere of its burrows [1–4]. Because of their adaptation to an underground life, mole rats have been used as a model species to study the physiological adjustment of mammals to a low O_2 (hypoxic) and high CO_2 (hypercapnic) environment [4]. However, despite the documentation of various physiological mechanisms that these animals seem to have evolved for adapting to a subterranean environment [2,4–12], only a few physicochemical aspects between mole rat and non-burrowing rodents have been studied in an effort to explain the underlying molecular mechanisms that may be responsible (cf. [8,12]). Comparisons of the hemoglobins between mole rats and white rats showed no major differences that might account for their adaptation to a hypoxic environment [8]. To our knowledge, no non-heme red cell proteins (NHP) have as yet been examined. Because red cell carbonic anhydrase plays such a pivotal role in acid-base balance and the transport and conversion of CO_2 ($CO_2+H_2O \Leftrightarrow HCO_3^- + H^+$), we decided to examine the carbonic anhydrases (CAs) of mole rat erythrocytes. Here, we report the surprising findings of the low-activity carbonic anhydrase isozyme, CA I, and a 56 kDa selenium-binding protein (SeBP) as the major NHPs of mole rat red blood cells. This appears to be the first report of a mammalian species whose red cells contain a selenium-binding protein as its major NHP, and CA I as its only carbonic anhydrase isozyme.

2. Materials and methods

2.1. Blood preparation

Whole blood samples were collected from five individual mole rats belonging to different populations across Israel (see [13] for map). Of the five mole rat populations, two (Kerem Ben Zima and Dalton) have the same chromosome number (2n = 52), whereas the rest possess different chromosome numbers (i.e. Quneitra, 2n = 54; Mukhraka, 2n = 58; Lahav, 2n = 60). After centrifugation of the uncoagulated blood, the packed red cells were washed with 0.85% NaCl, lysed with one volume of distilled H_2O, treated with toluene to remove stromata, and the hemoglobin removed with the ethanol/chloroform method as previously described [14–16]. The NHPs were concentrated in dialysis tubing under vacuum. Blood from a healthy white laboratory rat (*Rattus norvegicus*) was used as a control in our experimental procedures.

2.2. Electrophoresis

Proteins were separated in 12% vertical starch gels [14], and the cyanogen bromide (CNBr)-digested fragments separated in 10% SDS-PAGE gels. CA activity was detected in starch gels after electrophoresis using the bromothymol blue staining method, and the gels were further stained for proteins with 0.1% nigrosin [14,17].

2.3. Immunodiffusion analysis

Antibodies were prepared in rabbits against CA I and CA II purified from laboratory white rat red cells, and the mole rat NHPs tested against anti-rat CA I and CA II sera by immunodiffusion analyses on 0.4% agar plates with appropriate controls as previously described [17].

2.4. Chromatography

Reverse-phase high performance liquid chromatography (HPLC) was used to separate and then to quantify the NHPs. Standard buffer A (0.1% TFA) and buffer B (80% MeCN/0.1% TFA) were used in a 130 ABI HPLC apparatus to develop a 45–75% gradient over a retention time of 60 min. Major peaks were collected and then digested with 10 mg/ml CNBr in 70% TFA for 4 h.

2.5. Amino acid sequencing and phylogenetic analyses

Amino acid sequences from selected CNBr fragments were determined using an automatic protein sequencer (494 ABI). A BLAST search for these sequences was conducted against existing protein

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Abbreviations: CA I, CA II, carbonic anhydrases I and II; RBC, red blood cell; SeBP, selenium-binding protein; CNBr, cyanogen bromide; HPLC, reverse-phase high performance liquid chromatography; NHP, non-heme protein

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databases. Phylogenetic analyses were carried out with the MEGA computer program [18], and phylogenetic trees constructed using both UPGMA and neighbor-joining methods. To examine the robustness of the phylogenetic groupings, bootstrap tests with 1000 replica were performed.

2.6. Selenium assays

Packed RBCs (0.5 ml) prepared from two mole rats (Dalton and Quneitra) were used to measure total Se in erythrocytes by graphite furnace atomic absorption spectroscopy (GFAAS) at the laboratory of the National Medical Services (Willow Grove, PA). The same amount of RBCs from a laboratory white rat was used as controls.

3. Results

3.1. Immunodiffusion and protein analyses

After hemoglobin extraction, the remaining NHPs from all five mole rat samples showed similar patterns on starch-gel electrophoresis. Of the two major bands, only one showed CA activity (data not shown). In SDS-PAGE gel, two major bands were present at 30 and 55 kDa. The proteins from all five samples cross-reacted immunologically with rabbit anti-rat CA I sera, whereas no cross-reaction with anti-rat CA II was observed (Fig. 1).

The five samples showed similar HPLC profiles, revealing two major peaks at retention times around 20 and 28 min (Fig. 2). The protein peak at 28 min is about 1.3 times more abundant than the protein at 20 min. CNBr digestion of the proteins from the two major peaks collected from the same mole rat resulted in different peptide patterns, whereas peaks at similar retention times from different samples showed similar patterns (Fig. 3A). The CNBr digestion patterns of the smaller 20 min peak from different individuals resembled the typical CA I pattern with three major fragments at 10 kDa, 25 kDa, and 30 kDa. The CNBr digestion pattern of the major peak at 28 min resulted in several smaller peptides of <15 kDa, and did not resemble the digestion patterns of either CA I or CA II from other mammalian species (H. Yang, unpublished data).

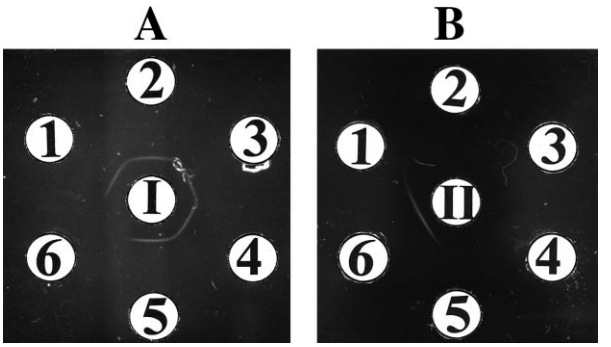


Fig. 1. Immunodiffusion cross-reaction patterns between rabbit anti-rat CA I and CA II sera and mole rat red cell non-heme proteins. Note absence of cross-reactivity with anti-CA II. Antisera in center wells: A (I = rat anti-CA I); B (II = rat anti-CA II); surrounding wells: 1–5 = mole rats from different populations; A6 = negative control; B6 = rat CA positive control.

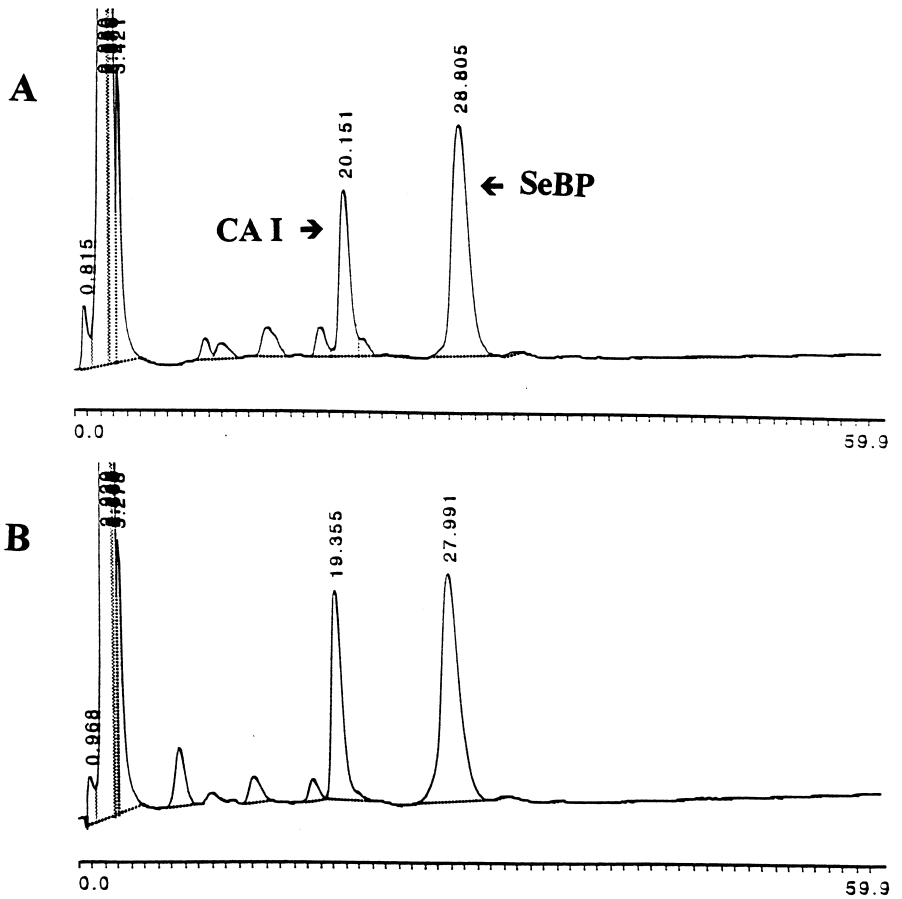


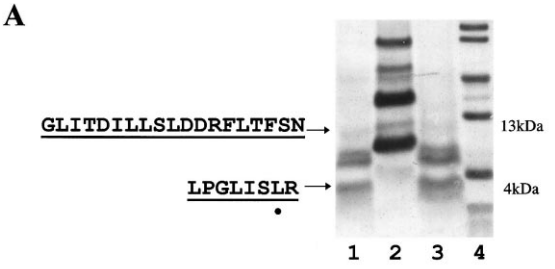
Fig. 2. HPLC profiles from two representative mole rats from population Dalton (A) and Lahav (B), showing major peaks identified as carbonic anhydrase I (CA I) and 56 kDa selenium-binding protein (SeBP). The two peaks from the Lahay mole rat (B) were not sequenced.

3.2. Carbonic anhydrase I

The amino acid sequence of a 10 kDa CNBr fragment from the smaller 20 min HPLC peak could be readily aligned with other CA isozyme sequences (Fig. 4A). The putative mole rat CA I sequence, corresponding to positions 148–165 (human CA I numbering) of other CA isozymes [19], shared three unique amino acid residues that are characteristic of other CA I sequences at positions 148, 154, and 155. Compared with the mouse CA I sequence, the mole rat sequence has substitutions at 150-Leu/Val, 151-Gln/Pro, and 156-Asn/Ser. Phylogenetic analysis using homologous CA I, CA II, and CA III sequences showed that the putative mole rat CA I sequence clustered with the CA I sequences from other species. Both UPGMA and neighbor-joining methods (with or without p-distance and Poisson correction) constantly produced phylogenetic trees with similar topologies. Although the 18 amino acid sequence that was compared is relatively short, when the shark CA sequence (a precursor of CA I–III) was used as the outgroup, the three major branches leading to CA I, CA II, and CA III clustered separately on the phylogenetic tree with significantly high bootstrap values of 98, 97, and 100%, respectively (Fig. 4B).

3.3. Identification of selenium-binding protein

Partial amino acid sequences of CNBr peptide fragments from each of the two HPLC protein peaks were determined from the Dalton mole rat. Sequencing the 4 and 18 kDa CNBr fragments from the 28 min peak produced sequences of eight and 19 amino acids (Fig. 3A). When a BLAST search was performed against existing protein databases, they matched different regions of the same mouse 56 kDa selenium-binding protein sequence deposited in the Swiss protein database (SWISS-PROT: P17563). The 19 amino acid sequence of the mole rat showed a 100% identity between positions 308 and 326 with the mouse 56 kDa SeBP 99 (Fig. 3B). The eight amino acid sequence obtained from the 4 kDa fragment matched the same mouse 56 kDa SeBP sequence between positions 96 and 103 with one amino acid difference (Ser/Leu) at position 102 (Fig. 3B).



B

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MATKCTKCGP GYSTPLEAMK GPREEIVYLP CIYRNTGTEA PDYLATVDVD 50
PKSPQYSQVI HRLPMPYLKD ELHHSGWNTC SSCFGDSTKS RNKLILPGLI 100
SSRIYVVDVG SEPRAPKLHK VIEASEIQAK CNVSSLHTSH CLASGEVMVS 150
TLGDLQGNGK GSFVLLDGET FEVKGTWEKP GDAAPMGYDF WYQPRHNVMV 200
STEWAAPNVF KDGFNPAHVE AGLYGSRIFV WDWQRHEIIQ TLQMTDGLIP 250
LEIRFLHDPS ATQGFVGCAS APNIQRFYKN AEGTWSVEKV IQVPSKKVKG 300
WMLPGVPGLI TDILLSLDDR FLYFSNWLHG DIRQYDISNP QKPRLAGQIF 350
LGGSIVRGGS VQVLEDQELT CQPEPLVVKG KRIPGGPQMI QLSLDGKRLY 400
ATTSLYSAWD KQFYPDLIRE GSMMLQIDVD TVNGGLKLNP NFLVDFGKEP 450
LGAALAHELR YPGGDCSSDI WI                              472
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Fig. 3. A: SDS gel showing CNBr digestion of the proteins from the two major peaks separated by HPLC in Fig. 2. 1 = Dalton peak at 28 min; 2 = Dalton peak at 20 min; 3 = Lahav peak at 27 min; 4 = M12 protein standard. Two partial protein sequences were obtained from 13 kDa and 4 kDa fragments of the Dalton peak at 28 min. The single amino acid substitution (102-S/L) is indicated by a dot. B: Complete sequence of mouse liver 56 kDa selenium-binding protein [35]; portions that were matched by sequence fragments from mole rat are marked in bold and underlined.

A

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                    148                 165
Mole Rat CA    MKLGQANPNLQKVLDALN
Mouse CA I     MKVGPANPSLQKVLDALN
Rabbit CA I    MKVGQANPKLQKVLDALS
Sheep CA I     MKVGQANPNLQKVLDALK
Human CA I     MKVGEANPKLQKVLDALQ
Mouse CA II    LKIGPASQGLQKVLEALH
Human CA II    LKVGSAKPGLQKVVDVLD
Rat CA II      LKIGPASQGLQKITEALH
Mouse CA III   LKIGREKGEFQILLDALD
Human CA III   LKIGHENGEFQIFLDALD
Rat CA III     LKIGREKGEFQILLDALD
Shark CA       MLVCDSNPAL-KIVCSLE
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B

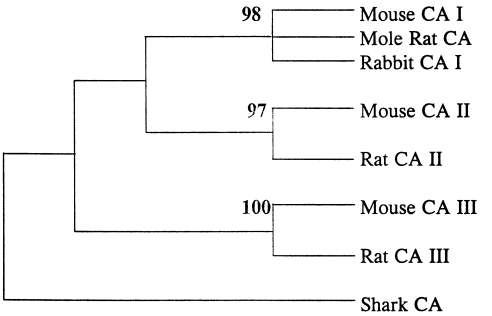

Fig. 4. A: Alignment of amino acid sequence of a CNBr fragment from 20 min HPLC peak (Fig. 2A) compared with the CA I, II and III amino acid sequences from other mammals and shark CA (cf. [19]). Position numbers based on human CA I sequence. B: Phylogenetic analysis of putative mole rat CA I sequence showing a UPGMA tree built using the above alignment; numbers in each node indicate 1000 replicate bootstrap values.

3.4. Red cell selenium analyses

GFAAS measurements of total Se levels in equal amounts (0.5 ml) of packed RBCs from one laboratory rat and two mole rat samples were: 220 μg/l Se in rat, and only 33 and 43 μg/l in the two mole rat samples. The mole rat levels are also considerably lower than the values of 220 μg/l (rat) to 950 μg/l (dolphin) reported from the red cells of other mammals (H. Yang, unpublished assays).

4. Discussion

Characteristically, both a low-activity CA I and a high-activity CA II isozyme are found in the erythrocytes of mammals. An exception to this is that only the high-activity CA II isozyme is present in the red cells of the cat family (Felidae) and in ruminants (e.g. ox, sheep, goat, cf. [20]). In humans, the CO_2 hydration activity of CA I is about 10 times lower than that of CA II whose turnover number of $\sim 10^6$ s^{-1} makes it one of the most active enzymes known [21]. As yet, no species of amniotes (reptiles, birds, and mammals) has been shown to lack the high-activity CA II isozyme in their red blood cells. When both CA I and CA II are present, the levels of CA I are usually greater than CA II. For example, in humans CA I and CA II levels are, respectively, ~13.0

and ~2.0 μg/mg Hb [17]. In rodents, however, the levels of CA II are often about equal to, or even greater than, CA I ([22]; R.E. Tashian, unpublished).

4.1. Carbonic anhydrase I

Evidence that CA I is the only CA isozyme in the mole rat red blood cells comes mainly from our findings that: (a) the hemolysates cross-reacted with anti-rat CA I, but not anti-rat CA II sera, (b) the 18 amino acid sequence of a CNBr fragment from the 20 min HPLC peak shares three residues that are unique to the CA I sequences of other mammals, but not found in the corresponding sequences of any other CA isozyme (i.e. CA II–VII) (Fig. 4A; [19]), and (c) the clustering (after phylogenetic analyses) of a portion of the mole rat putative CA I amino acid sequence with the corresponding sequence CA I sequence from other species.

The finding of a mammal that characteristically does not express any red cell CA II clearly shows that CA I alone is capable of carrying out the transport of metabolic CO_2 to the lungs via the reversible hydration of $CO_2 \Leftrightarrow HCO_3^-$. This feature was predicted by the finding that humans and mice with CA II deficiency mutations seemingly showed no adverse respiratory effects under normal conditions [23–25]. A recent study of respiratory acidosis in mutant CA II-deficient mice showed them to have a partial respiratory compensation for metabolic acidosis indicating a mixed respiratory and metabolic acidosis due to CA II deficiency in red cells and pneumocytes [26]. A report on comparisons of respiratory systems between mole rats and white rats has shown that structural differences in lung and muscle of mole rats might account for their higher O_2 diffusing capacity to achieve high metabolism underground [7]. It has also been reported that compared to the white rat, the urine of the mole rat shows higher levels of calcium and magnesium bicarbonate suggesting that this may be a means for the mole rat to reduce its CO_2 in a hypercapnic environment [12].

Why would the absence of the high-activity, CA II isozyme in the red cells of the mole rat be advantageous? One possibility is that the respiratory acidosis produced by the increased CO_2 in the blood stimulates more rapid breathing thereby taking up more O_2 from the hypoxic environment. If the high-activity CA II was present, it would tend to lower the P_{CO_2}, thereby countering the adaptive effects of the increased breathing. This may also be the reason that the administration of CA inhibitors to dogs and humans can assist in their accommodation to high altitude [27,28]. Another advantage might be that the respiratory acidosis would produce a left-shifted Hb-O_2 dissociation curve that would facilitate O_2 release to the tissues [29–31]. A further possibility is that the high ventilatory drive produced by the inefficient removal of CO_2 would be useful in regulating body temperature when the temperatures of the underground burrows reach high levels. Obviously, critical comparative physiological experiments under hypoxic stress must be undertaken between mole rats and non-subterranean rodents in order to gain insights into the physiological aspects of their adaptations. It should be noted that the above suggestions are based on the assumption that the mole rat CA I possesses the characteristic low activity of CA I isozymes, and this has not yet been demonstrated.

4.2. Selenium-binding protein

In mammals, the trace element selenium (Se) is an essential nutrient, with highest concentrations (humans) in kidney, liver, and spleen. In humans and other mammals a number of abnormalities are known to result from Se deficiency. Se is also known to inhibit cell growth, and Se-supplemented diets appear to reduce the incidence of a variety of cancers. A number of selenoproteins, some of which are enzymes, have been identified in mammals [32–34], ranging in molecular weight from 10 to >90 kDa. In mammals, these SeBPs have been found in almost every tissue; however, as yet no Se-BP been detected in erythrocytes, with the exception of glutathione peroxidase which is present at very low levels [32–34]. Thus, detection of a 56 kDa SeBP as the major non-heme protein in the red cells of the mole rat was a rather surprising finding.

Orthologues of the 56 kDa SeBP had previously been reported from human and mouse liver cDNAs [35,36]. The derived proteins are 472 amino acids in length and the human and mouse sequences are highly conserved with an identity of 87%. The total 27 amino acid residues in the two mole rat fragments differed by only one residue from the mouse sequence. It would thus appear that the mouse and mole rat 56 kDa SeBPs would be highly conserved which could indicate an important physiological function that might involve binding to cellular proteins or structures.

The unusually low concentration of Se that we detected in mole rat RBCs suggests that the expression of 56 kDa SeBP in the red cells might play a role in the tissue transport or storage of Se. Since the mole rats samples were collected from different populations with different soil types across Israel [37], it is unlikely that the low Se and high SeBP in mole rat RBCs simply reflect an abnormal geochemical distribution in the local environment. It is also conceivable that the 56 kDa SeBP may interact with hemoglobin or other RBC proteins to alter O_2 or CO_2 binding affinities.

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