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Genetics

A carbonic anhydrase from the nacreous layer in oyster pearls

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ABSTRACT **It is believed that the polymorphism observed in calcium carbonate crystals, such as aragonite and calcite in mollusk shells, is controlled by organic matrix proteins secreted from the mantle epithelia. However, the fine structures of these proteins are still unknown, and to understand the molecular mechanisms of mineralization process, detailed structural analyses of the organic matrix proteins are essential. For this, we have carried out purification, characterization, and cDNA cloning of nacrein, which is a soluble organic matrix protein in the nacreous layer of oyster pearls. Northern blot analysis showed that the nacrein transcript was specifically expressed in mantle pallial. Analysis of the deduced amino acid sequence revealed that the protein contained two functional domains: one was a carbonic anhydrase and another was a Gly-Xaa-Asn (Xaa = Asp, Asn, or Glu) repeat domain; however, the carbonic anhydrase domain was split into two subdomains with insertion of the Gly-Xaa-Asn repeat domain between them. Our findings suggest that nacrein actually functions as a matrix protein whose repeated Gly-Xaa-Asn domain possibly binds calcium and as a carbonic anhydrase that catalyzes the HCO_3^- formation, thus participating in calcium carbonate crystal formation of the nacreous layer.**

Crystallization, observed in many organisms, is a genetically regulated process. In the mammal, several factors regulating bone morphogenesis are identified. Osteopontin, an acidic calcium binding protein, is isolated from the mineralized phase of bone (1). Another bone matrix protein, osteocalcin (2), contains γ-carboxyglutamic acid residues, which participate in calcium binding. In general, the mollusk shell is mainly composed of two layers, a prismatic layer and nacreous layer. Both layers are in the forms of calcium carbonate crystal; however, the prismatic layer forms calcite and the nacreous layer forms aragonite. Such crystal polymorphism in the two closely situated layers is a highly elaborated phenomenon in the mollusk. In such regulated processes, organic matrices secreted from the mantle epithelia have been suggested to play critical roles (3); the major components of soluble organic matrices are aspartic acid-rich calcium binding proteins (4–8). Recently, Falini *et al.* (9) and Belcher *et al.* (10) reported that macromolecules extracted from the nacreous shell layers induced aragonite formation *in vitro*.

Pearl oysters (*Pinctada fucata*) produce pearls inside the shells. Such a pearl is equivalent to the mollusk shell layer, which is composed of the aragonite crystal and organic matrices. Therefore, to isolate the organic matrix protein, we have chosen the pearl as a starting material. Staining with Coomassie brilliant blue and Stains-all (Nacalai Tesque, Kyoto), we have identified a 60-kDa protein that we named nacrein. Analysis of cDNA encoding nacrein revealed that it had a similar domain to carbonic anhydrase (CA). Furthermore, the native nacrein purified from the pearl possessed CA enzymatic activity. To our knowledge, this is the first report of the amino acid sequence of a molluscan organic matrix protein.

MATERIALS AND METHODS

Purification of the Organic Matrix Protein Nacrein. Nacreous layers of *P. fucata* pearls were crushed to a fine powder. The powder (20 g) was extracted with 100 ml of 0.5 M EDTA (pH 8.0) containing 0.01% sodium azide. Extraction was performed at room temperature with continuous stirring. After 3 days, the EDTA-soluble pearl extract was obtained by centrifugation at 30,000 × *g* for 20 min. The supernatant solution (125 ml) was diluted with an equal volume of water. The diluted solution (250 ml) was concentrated by ultrafiltration with a minimodule. The concentrated fraction (100 ml) was dialyzed against 1 liter of 1 mM EDTA (pH 7.0) solution overnight. The dialyzed fraction (250 ml) was concentrated to 25 ml and subjected to ammonium sulfate fractionation. The precipitate by 80% saturation was dissolved in 4 ml of 10 mM EDTA (pH 7.0) and then dialyzed against the same buffer (1 liter) overnight. The dialyzed material (8.5 ml) was loaded onto a DEAE–Sephacel column (1.5 × 12 cm) previously equilibrated with 10 mM EDTA (pH 7.0). Then, the column was washed with the same buffer and eluted with a linear gradient of 0–0.8 M NaCl in the same buffer (total volume, 400 ml). Fractions of 8 ml were collected, and their protein concentrations were determined by trichloroacetic acid conductivity (11). In this procedure for protein quantification, protein is precipitated by trichloroacetic acid, and the turbidity at 340 nm was measured after 30 min. The major peak fractions (0.4 M NaCl) on DEAE–Sephacel were pooled (80 ml) and dialyzed against 10 mM EDTA (pH 7.0) overnight. After dialysis, the material was loaded onto a DEAE–Sephacel column and then eluted with 0.8 M NaCl, followed by concentration to 2 ml by ultrafiltration. The concentrated material was loaded on a Sephadex G-75 column (2 × 95 cm) previously equilibrated with 10 mM EDTA (pH 7.0) containing 0.2 M NaCl and eluted at a flow rate of 12.5 ml/h with the same buffer.

Molecular Analyses. The peak fraction on Sephadex G-75 was separated by SDS/PAGE (12), transferred to a polyvinylidene difluoride membrane and subjected to N-terminal amino acid sequence analysis. Degenerate oligonucleotides [5′-C(G/T)IACICC(A/G)TC(A/G)TCCAT(A/G)TA(A/G)T(C/G)(A/G)TC(A/C/G)(A/T)(G/T)(C/T)TT(A/G)AACAT(A/G)TGI(C/G)(A/T)-3′] were synthesized and used as probes for cDNA screening. Total RNA was isolated by a single-step method (13) from the mantle pallial of *P. fucata*, and poly(A)$^+$ RNA was prepared by oligo(dT)-cellulose affinity chromatography using a mRNA purification

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Abbreviation: CA, carbonic anhydrase.

Data deposition: The sequence reported in this paper has been deposited in the GenBank data base (accession no. D83523).

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kit (Pharmacia). Poly(A)$^+$ RNA (1 μg) was subjected to cDNA library construction using a TimeSaver cDNA synthesis kit (Pharmacia). Five positive λ phage clones were isolated, and the longest cDNA was sequenced by the chain termination method (14) using an automated DNA sequencer (Applied Biosystems). Poly(A)$^+$ RNA (2 μg) was separated on a 1% agarose gel containing formaldehyde as described (15). Following transfer to a Nitroplus 2000 (Micron Separations, Westboro, MA), the filter was hybridized in 6× SSC, 5× Denhardt's solution, 0.5% SDS, 4 mM EDTA, 100 μg of salmon sperm DNA per ml, and digoxigenin-labeled probes at 65°C. The filter was washed in 0.5× SSC at 60°C and then visualized with a chemiluminescent substrate disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2′-(5′-chloro)tricyclo-[3.3.1.1^{3,7}]decan}-4-yl)phenyl phosphate (CSPD) according to the manufacturer's instructions (Boehringer Mannheim).

CA Assay. We expected the nacrein protein to be free of zinc, since the powdered pearl was decalcified with 0.5 M EDTA for 4 days followed by dialysis against zinc-free buffer. Thus, to examine the CA activity, the purified protein was dialyzed against 20 mM Tris·HCl (pH 7.0) buffer containing 2 mM $ZnCl_2$ and against the same buffer without $ZnCl_2$ to remove free zinc ions. We measured CA activity using the CO_2-Veronal indicator method (16, 17) as follows. Six drops of phenol red, 3 ml of 20 mM Veronal buffer (pH 8.3), and 20 μl of the test material-containing solution are mixed and placed in ice water; then the reaction was started by addition of 2 ml of ice-cold water saturated with CO_2 followed by observation of the time for the pH to drop to 7.3. Definition of unit is as follows: unit = $(T_0 - T)/T$, where T and T_0 are the reaction times required for the pH change from 8.3 to 7.3 at 0°C with and without a catalyst, respectively.

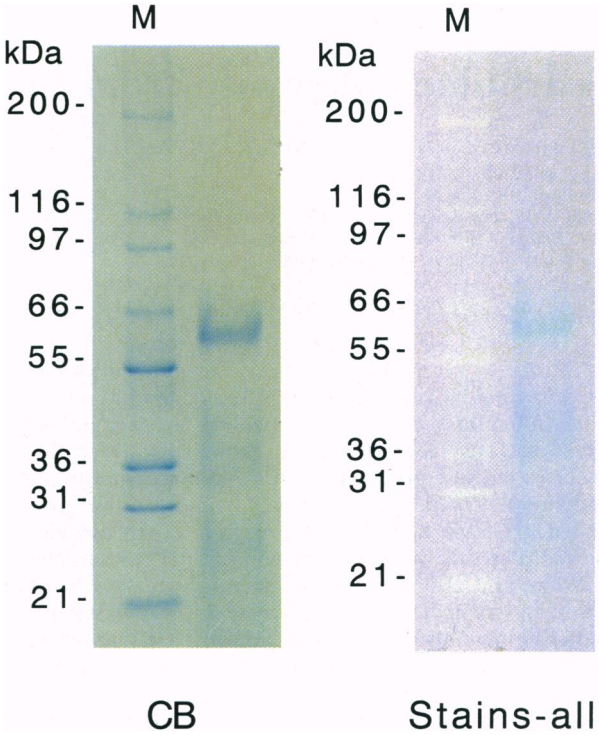

FIG. 1. Purification and characterization of the organic matrix protein nacrein. The major peak fraction on DEAE–Sephacel was separated on an SDS/polyacrylamide gel and visualized by Coomassie brilliant blue staining (CB) or stained with Stains-all. M, molecular mass standards.

RESULTS

Nacreous layers of pearls of *P. fucata* were ground to a powder, and the extracted organic matrix components were separated by DEAE–Sephacel ion-exchange chromatography. The major peak was analyzed by SDS/PAGE (12), which revealed a major 60-kDa protein (nacrein) (Fig. 1). Since previous reports indicated that a component of the organic matrix is an aspartic acid-rich calcium binding protein (4–8), we stained the same fraction with Stains-all as described (18). Stains-all is a cationic carbocyanine dye, which has been shown to stain calcium binding protein blue. As shown in Fig. 1, only the 60-kDa nacrein was stained blue, suggesting the protein is a calcium binding protein. Nacrein was further purified by Sephadex G-75 chromatography. The purified protein was separated by SDS/PAGE, transferred to a polyvinylidene difluoride membrane, and then subjected to N-terminal amino acid sequence analysis. The resulting amino acid sequence was (A/S)(S/H)MFKHDHYMDDGVR. Degenerate synthetic oligonucleotides based on the N-terminal amino acid sequence were used as probes for screening a cDNA library derived from mantle pallial cells, which are involved in the nacreous layer formation. A cDNA clone (λN6) was isolated and appeared to be almost full-length when compared with the endogenous poly(A)$^+$ RNA by Northern blot analysis (see Fig. 3). λN6 cDNA was 2.4 kb long and contained an open reading frame encoding a polypeptide of 447 aa (Fig. 2*a*). Residues 18–32 in the deduced amino acid sequence agreed with the N-terminal sequence of the isolated nacrein, except for residue 29, which was asparagine. The deduced amino acid residues 1–17 showed a typical signal sequence. If the N-terminal amino acid residue of nacrein corresponds to alanine at residue 18 of the deduced amino acid sequence, the calculated molecular mass could be 48 kDa. This value was slightly smaller than that of nacrein estimated by SDS/PAGE. Although the open reading frame of λN6 cDNA contained two possible N-glycosylation sites, there was no electrophoretic mobility shift of nacrein upon treatment with endoglycosidase H or F (data not shown). Therefore, other posttranslational modifications such as sulfation and phospholylation may have occurred to nacrein. In fact, sulfated groups are present at the nucleation site of mollusks (19, 20), and some bone matrix proteins are phosphorylated (1). An antiserum generated against the native nacrein derived from pearls recognized recombinant protein expressed in *E. coli* by using the cDNA (data not shown). This result indicates that λN6 cDNA certainly encodes the nacrein polypeptide.

Analysis of the nacrein amino acid sequence deduced from the DNA sequence showed the presence of Gly-Xaa-Asn repeats (Xaa = Asp, Asn, or rarely Glu) at residues 242–322. These may be sites for interaction with calcium. A search of the Swissprot protein data base (October 1995) revealed homology between nacrein and CA (Fig. 2 *a* and *b*). CA domain of Nacrein exhibited greatest identity to human CAII (refs. 21 and 22; 28% identical over their entire length). In particular, among the 36 residues of the active site (ref. 23; see Fig. 2*a*), 63% identity to human CAII was observed. Furthermore, nacrein retained the three histidines involving zinc-binding residues at positions 149, 151, and 174.

CA is a widespread zinc metalloenzyme that catalyzes the following interconversion between CO_2 and HCO_3^-: $CO_2 + H_2O \Leftrightarrow HCO_3^- + H^+$. We investigated whether nacrein had CA activity. As shown in Table 1, although the specific activity was relatively lower than that of bovine erythrocyte CA used as a standard, the purified nacrein protein on Sephadex G-75 possessed easily detectable CA activity. The CA activity of both nacrein and the bovine erythrocyte enzyme was inactivated by heat treatment (at 100°C for 10 min). A large amount of BSA, which is known not to have CA activity, also showed no activity. To eliminate the possibility of contamination, we excised the nacrein band from an SDS/polyacrylamide gel, and extracted the pure nacrein protein. The extract likewise indicated the CA activity (data not shown). These results clearly

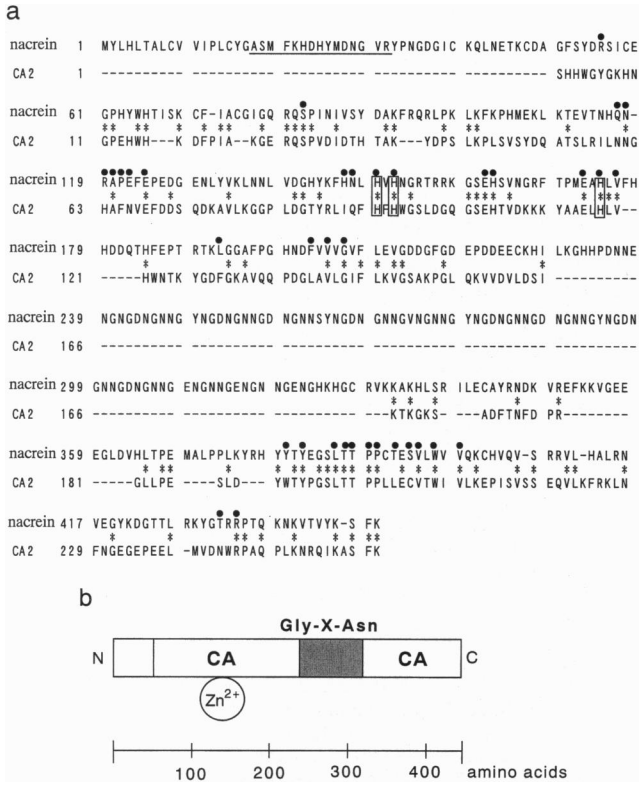

FIG. 2. (*a*) Comparison of the deduced amino acid sequence of nacrein and human CA II (CA2). Identical amino acids are indicated by asterisks. Thirty-six residues of the active site are marked by closed circles and three zinc binding histidine residues are boxed. The residues determined by protein sequencing are underlined. (*b*) Schematic representation of the nacrein protein sequence. CA homologous regions (CA) and the Gly-Xaa-Asn repeat domain (Gly-X-Asn) are indicated. The numbers indicate amino acid residue positions.

show that nacrein is not only a structural protein but also a catalyst.

It is believed that organic matrix proteins are secreted from the mantle epithelium. Because of this, in Fig. 3, we further investigated expression of the nacrein mRNA in the mantle. The 2.4-kb nacrein mRNA was detected at extremely high levels in the mantle pallial, which contributes to nacreous layer formation. In contrast, in the mantle edge, which contributes to the prismatic layer formation, the nacrein mRNA was rarely detected.

DISCUSSION

Nacrein constitutes a significant proportion in the soluble matrix proteins of the nacreous layer. It is widely believed that aspartic acid-rich calcium binding proteins are responsible for

Table 1. Assay of carbonic anhydrase activity

Sample	Amount used for assay, μg	*T*, sec	Specific activity, units/mg
—	—	105 (=T_0)	—
BECA	0.1	30	2.5×10^4
BECA (heat-inactivated)	0.1	130	—
Nacrein	0.8	45	1.6×10^3
Nacrein	1.6	24	2.1×10^3
Nacrein (heat-inactivated)	0.8	140	—
BSA	4.0	120	—

BECA, bovine erythrocyte CA; BSA, bovine serum albumin. Each value shown is an average of two independent experiments.

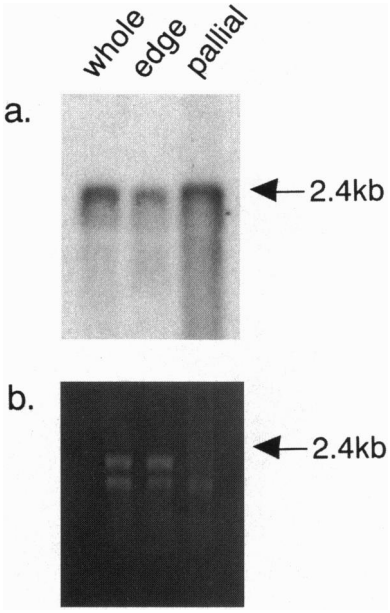

FIG. 3. Northern blot analysis of the nacrein transcripts. (*a*) Poly(A)+ RNAs prepared from whole mantle, the mantle edge, and the mantle pallial were subjected to Northern blot analysis with λN6 cDNA probe. (*b*) The same samples stained with ethidium bromide.

biomineralization in mollusk shells (4–8). The regularly spaced carboxyl groups of Asp or Glu side chains on the surface of such calcium binding protein participate directly in calcium binding, either alone or in concert with other Asp or Glu in the polypeptide, such as osteopontin (24). Furthermore, the malonic acid moiety of the β-carboxyaspartic acid or γ-carboxyglutamic acid side chain could also participate in calcium binding (2). Nacrein has an acidic Gly-Xaa-Asn (Xaa = Asp, Asn, or Glu) domain and was stained blue with Stains-all, which suggests that nacrein is a calcium binding protein. Probably, nacrein is the major aspartic acid-rich calcium binding protein in the nacreous layer. The Gly-Xaa-Yaa (Yaa = any amino acid) repeats like those in the collagenous domain are also found in a structural protein of the inner ear, which produces calcium carbonate crystals (25). Possibly, forming three-dimensional lattices or meshworks like collagen molecules, the Gly-Xaa-Asn repeats could contribute to crystal growth.

Two different forms of calcium carbonate crystal exist in a shell: one is calcite in the prismatic layer and another is aragonite in the nacreous layer. The major difference between these forms is the position of the carbonate groups. Addadi, Weiner, and colleagues (26, 27) have already demonstrated that acidic proteins from shells regulate biological calcite growth. Moreover, Falini *et al.* (9) and Belcher *et al.* (10) showed that acidic macromolecules extracted from the prismatic and nacreous shell layers induced calcite and aragonite formation, respectively, *in vitro*. These results suggest that the macromolecules modulate the stereochemical position of carbonate groups in the process of biological calcium carbonate crystallization.

The mantle of mollusk shellfish is known to contain CA activity (28). However, the exact nature of acidic proteins and CA activity in the formation of prismatic or nacreous layer is not well understood. We have shown that this CA activity results from the nacrein protein itself secreted from the mantle. CA domain of nacrein is the most similar to human CAII (28% identical) and 23–27% identical to human CAI, III, IV, V, and VI. CAIIs of chick, mouse, rat, and rabbit show 26–28% identity. These amino acid sequence homologies

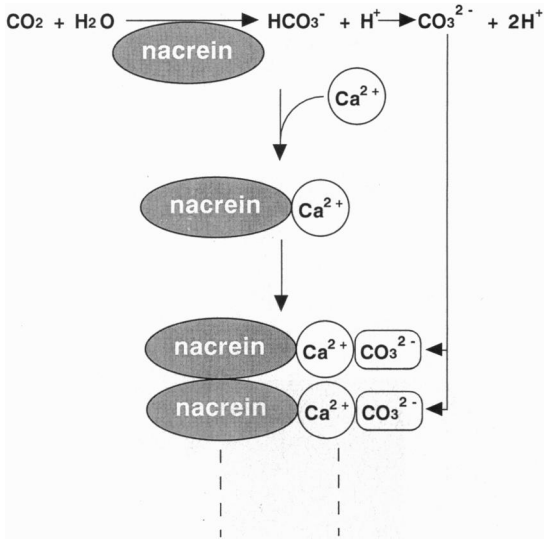

FIG. 4. A model for the nacreous layer formation by nacrein. See the text for details.

indicate that nacrein is more similar to CAII than different isozymes.

We propose the following model on the nacreous layer formation by the nacrein protein in the shell. CO_2 produced by respiration is converted to HCO_3^- in the presence of H_2O by nacreins. After binding of calcium ions, nacrein modulates the stereochemical position of carbonate groups dissociated from HCO_3^- to generate aragonite crystals (Fig. 4). Since calcium carbonate is soluble at acidic pH, it may be that the protons produced by the enzymatic reaction are diluted in seawater or removed with a proton pump. In this scheme, for simplicity, we did not consider the presence of some other factors, such as chitin and silk fibroin-like protein (9), both of which might cooperate with nacrein to produce a specific type of crystal in extracellular space between the mantle and shells. Further precise functional analyses of nacrein on nucleation and epitaxial growth of the aragonite crystal may lead to deeper understanding of various biomineralization processes by many organisms.

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