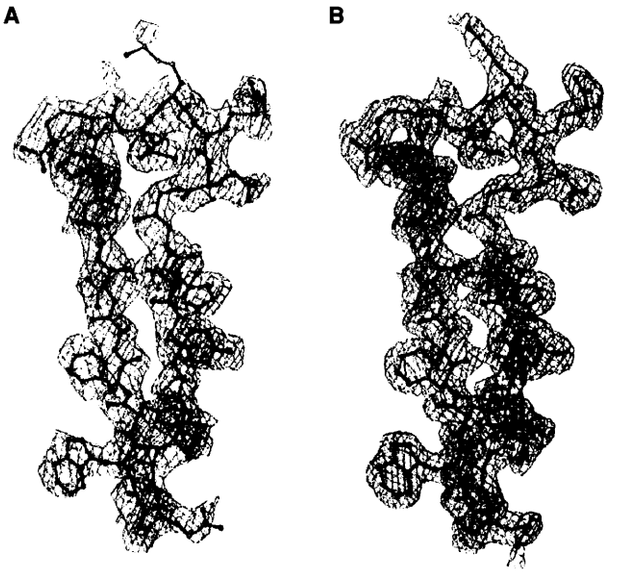

Fig. 4. (**A**) Multiple isomorphous replacement map, including anomalous dispersion, calculated to 3.0 Å resolution and contoured at 1σ showing residues 169 to 190, which form two antiparallel strands connected by a turn. (**B**) Map with coefficients $2F_{O,Nat} - F_c$ calculated to 2.4 Å resolution and contoured at 1σ showing the same region of secondary structure as in (A). The structure amplitudes, F_c, and the phases for the map were calculated from the current refined model (Table 1). Created with the program O (*28*).

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25–Hydroxyvitamin D_3 1α-Hydroxylase and Vitamin D Synthesis

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Renal 25–hydroxyvitamin D_3 1α-hydroxylase [1α(OH)ase] catalyzes metabolic activation of 25–hydroxyvitamin D_3 into 1α,25–dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$], an active form of vitamin D, and is inhibited by 1α,25$(OH)_2D_3$. 1α(OH)ase, which was cloned from the kidney of mice lacking the vitamin D receptor (VDR$^{-/-}$ mice), is a member of the P450 family of enzymes (P450$_{VD1α}$). Expression of 1α(OH)ase was suppressed by 1α,25$(OH)_2D_3$ in VDR$^{+/+}$ and VDR$^{+/-}$ mice but not in VDR$^{-/-}$ mice. These results indicate that the negative feedback regulation of active vitamin D synthesis is mediated by 1α(OH)ase through liganded VDR.

Vitamin D is metabolized by sequential hydroxylations in the liver and kidney to a family of seco-steroids. The two most biologically active forms of vitamin D are 1α,25$(OH)_2D_3$ and 24R,25–dihydroxyvitamin D_3 [24R,25$(OH)_2D_3$] (*1*, *2*). The binding of 1α,25$(OH)_2D_3$ to the nuclear receptor for the hormonally active form of vitamin D (VDR) activates the VDR (*3*), with subsequent regulation of physiological events such as calcium homeostasis and cellular differentiation and proliferation (*4*). Hydroxylation of 25–hydroxyvitamin D_3 [25$(OH)D_3$] is mediated by 25$(OH)D_3$ 1α-hydroxylase [1α(OH)ase] in the proximal tubule of the kidney. 1α(OH)ase is inhibited by its end product, 1α,25$(OH)_2D_3$ (*5*), and activated by calciotropic peptide hormones such as calcitonin and parathyroid hormone (*6*, *7*). Thus, serum concentrations of 1α,25-$(OH)_2D$ are kept constant. Vitamin D–dependent rickets type I (*8*) may be caused by mutations in the 1α(OH)ase gene. Biochemical analysis of semipurified 1α-(OH)ase protein has suggested that 1α(OH)ase belongs to the P450 family of enzymes (*9*).

We developed a nuclear receptor–mediated expression system to clone the cDNA encoding 1α(OH)ase. This system is based on the fact that a precursor of 1α,25$(OH)_2D_3$, 25$(OH)D_3$, can activate the transactivation function of the VDR only in the presence of 1α(OH)ase activity. Mice lacking the VDR (VDR$^{-/-}$ mice) developed an abnormally high serum concentration of 1α,25$(OH)_2D$ at 7 weeks, suggesting excessive 1α(OH)ase

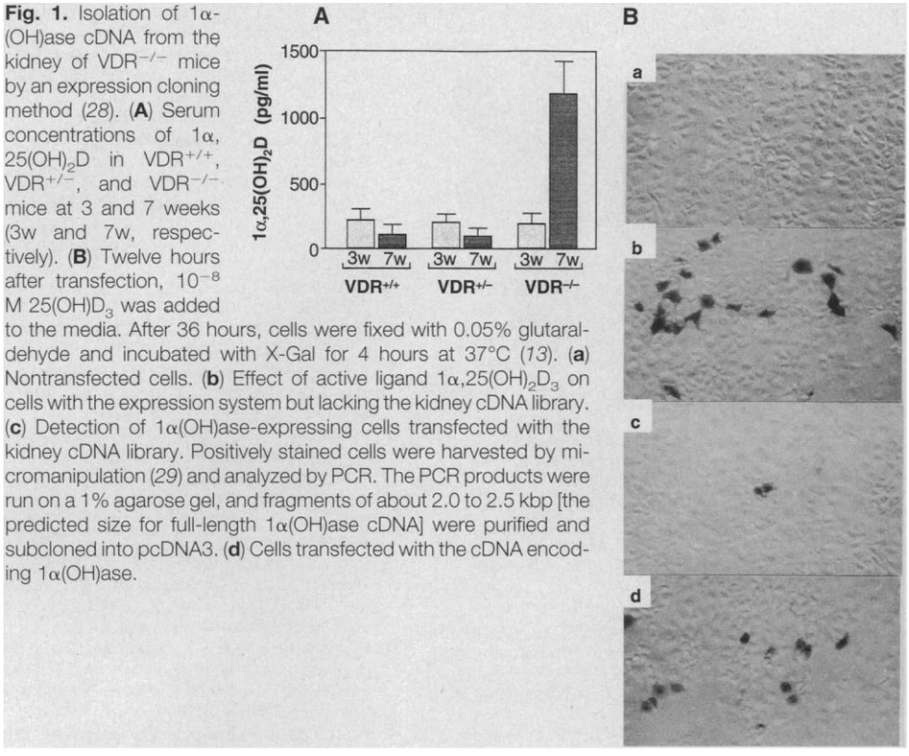

Fig. 1. Isolation of 1α-(OH)ase cDNA from the kidney of $VDR^{-/-}$ mice by an expression cloning method (*28*). (**A**) Serum concentrations of 1α, $25(OH)_2D$ in $VDR^{+/+}$, $VDR^{+/-}$, and $VDR^{-/-}$ mice at 3 and 7 weeks (3w and 7w, respectively). (**B**) Twelve hours after transfection, 10^{-8} M $25(OH)D_3$ was added to the media. After 36 hours, cells were fixed with 0.05% glutaraldehyde and incubated with X-Gal for 4 hours at 37°C (*13*). (**a**) Nontransfected cells. (**b**) Effect of active ligand $1\alpha,25(OH)_2D_3$ on cells with the expression system but lacking the kidney cDNA library. (**c**) Detection of 1α(OH)ase-expressing cells transfected with the kidney cDNA library. Positively stained cells were harvested by micromanipulation (*29*) and analyzed by PCR. The PCR products were run on a 1% agarose gel, and fragments of about 2.0 to 2.5 kbp [the predicted size for full-length 1α(OH)ase cDNA] were purified and subcloned into pcDNA3. (**d**) Cells transfected with the cDNA encoding 1α(OH)ase.

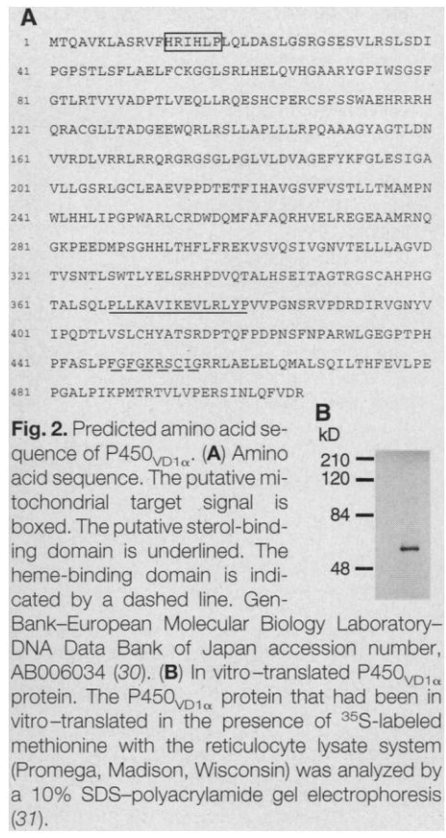

Fig. 2. Predicted amino acid sequence of $P450_{VD1\alpha}$. (**A**) Amino acid sequence. The putative mitochondrial target signal is boxed. The putative sterol-binding domain is underlined. The heme-binding domain is indicated by a dashed line. GenBank–European Molecular Biology Laboratory–DNA Data Bank of Japan accession number, AB006034 (*30*). (**B**) In vitro–translated $P450_{VD1\alpha}$ protein. The $P450_{VD1\alpha}$ protein that had been in vitro–translated in the presence of ^{35}S-labeled methionine with the reticulocyte lysate system (Promega, Madison, Wisconsin) was analyzed by a 10% SDS–polyacrylamide gel electrophoresis (*31*).

activity (Fig. 1A) (*10*). Kidneys from these mice were used to prepare an expression library with the expression vector pcDNA3 in COS-1 cells. These cells were then transfected with another vector expressing a chimeric protein that includes the VDR ligand-binding domain [VDR(DEF)] fused to the yeast GAL4 DNA-binding domain [GAL4-VDR(DEF)] (*11*) and with a reporter plasmid bearing *lacZ* regulated by the GAL4 binding site (17M2-G–*lacZ*). Vectors expressing adrenodoxin (ADX) and adrenodoxin reductase (ADR) were also included to support efficient hydroxylation (*12*). When supplied with 25-$(OH)D_3$, cells expressing 1α(OH)ase would produce ligands that are able to activate GAL4-VDR(DEF) and would be identifiable by expression of β-galactosidase (β-Gal) (Fig. 1Bc) (*13*). Transfected plasmids were extracted from eight β-Gal–positive cells and subjected to polymerase chain reaction (PCR) for amplification of the inserted cDNAs. PCR products of 2.0 to 2.5 kbp (the expected size for P450 family gene transcripts) (*14*) were recovered and subcloned. Sequence analysis of the isolated cDNAs from 64 random clones revealed that 13 clones encoded an identical, complete open reading frame (ORF). Reintroduction of this single cDNA sequence rendered the cells positively stained (Fig. 1Bd).

Using this cDNA as a probe, we obtained the full-length cDNA by colony hybridization screening of the same library. The amino acid sequence derived from the ORF predicts a protein with 507 amino acids (Fig. 2A). The in vitro–translated protein is 55 kD (Fig. 2B), which is similar in size to semipurified 1α(OH)ase (*9*). This protein (hereafter designated $P450_{VD1\alpha}$) has a mitochondrial target signal and is homologous to members of the P450 family (*14*), particularly to rat vitamin D_3 25–hydroxylase (41.7%) and mouse $25(OH)D_3$ 24–hydroxylase [24(OH)ase] (31.6%) (*15*, *16*). The putative sterol-binding domain (93% and 60% for rat and mouse, respectively) (*17*) and the heme-binding domain (70% and 80%, respectively) (*18*) show the greatest sequence similarities.

To confirm that $P450_{VD1\alpha}$ can convert $25(OH)D_3$ into $1\alpha,25(OH)_2D_3$, which in turn activates the VDR, we transfected COS-1 cells with GAL4-VDR(DEF), chloramphenicol acetyltransferase (CAT) reporter plasmid (17M2-G–CAT) (*19*), ADX and ADR expression vectors (*12*), and the $P450_{VD1\alpha}$ expression vector. Activation by $25(OH)D_3$ was observed only in the presence of $P450_{VD1\alpha}$, ADX, and ADR (Fig. 3A). These results indicate that $P450_{VD1\alpha}$ is 1α(OH)ase that converts $25(OH)D_3$ into $1\alpha,25(OH)_2D_3$.

To chemically confirm the enzymatic product of $P450_{VD1\alpha}$, we used normal and reversed phases of high-performance liquid chromatography (HPLC) (*20*). When ^3H-labeled $25(OH)D_3$ was added to cells transfected with the $P450_{VD1\alpha}$ expression vector, a metabolite was detected in the incubated medium. The retention times of the metabolite matched those of authentic $1\alpha,25(OH)_2D_3$ in both the normal and reversed phases of HPLC (Fig. 3B), even when different HPLC systems were used on both phases (*21*, *22*).

In both normal and $VDR^{-/-}$ mice, the 2.4-kbp $P450_{VD1\alpha}$ transcript was detected only in the kidney at 7 weeks (Fig. 4A). We did not detect 1α(OH)ase transcript in the other tissues, although 1α(OH)ase activity has been reported in extrarenal tissues such as placenta and macrophages (*1*, *22*, *23*). $P450_{VD1\alpha}$ was overexpressed in the $VDR^{-/-}$ mice, by about 2.5-fold at 3 weeks and 50-fold at 7 weeks (Fig. 4, B and C). Administration of $1\alpha,25(OH)_2D_3$ repressed 1α(OH)ase gene expression in the $VDR^{+/+}$

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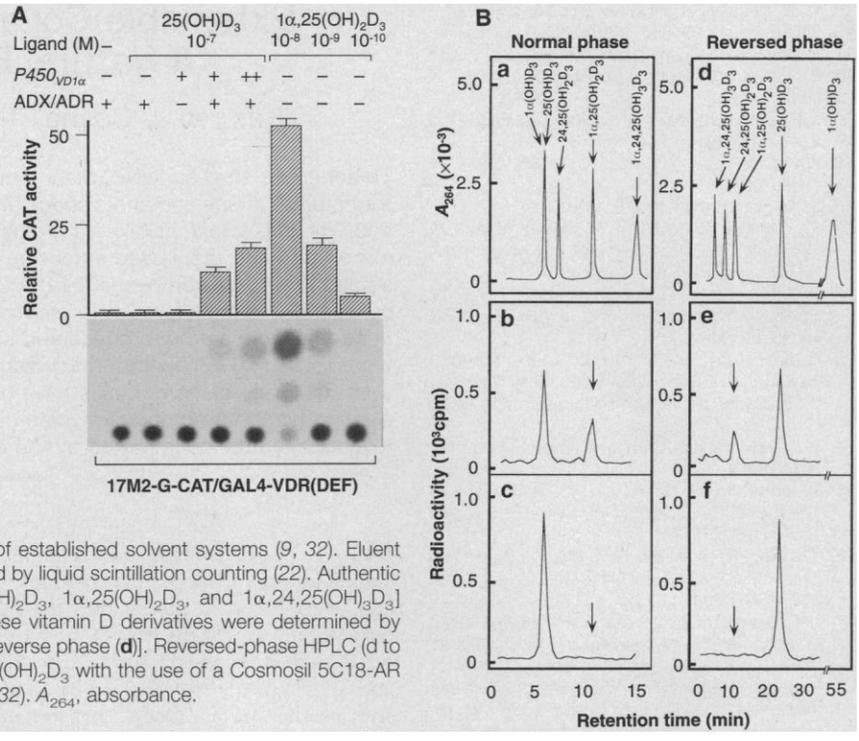

Fig. 3. Conversion of $25(OH)D_3$ into an active vitamin D_3 serving a VDR ligand by $P450_{VD1\alpha}$ (**A**) and identification of the converted $25(OH)D_3$ by HPLC analysis (**B**). (A) COS-1 cells were cotransfected with 0.5 μg of GAL4-VDR(DEF), 1 μg of 17M2-G–CAT, 0.5 μg each of the ADX and ADR expression vectors, and 1 μg (+) or 3 μg (++) of the $P450_{VD1\alpha}$ expression vector with or without the indicated ligands (*19*). One representative CAT assay (lower panel) and relative CAT activities (upper panel) corresponding to means ± SEM for three independent experiments are shown. (B) Normal- and reversed-phase HPLC analysis of $25(OH)D_3$ metabolite converted by $P450_{VD1\alpha}$. ^{3}H-Labeled $25(OH)D_3$ (10^5 dpm; 6.66 terabecquerels/mmol) (Amersham International) was incubated with COS-1 cells transfected with (**b** and **e**) or without (**c** and **f**) the $P450_{VD1\alpha}$ expression vector together with ADX and ADR expression vectors for 6 hours at 37°C. The cultured media were extracted with chloroform-methanol and analyzed on normal-phase HPLC (a to c) with TSK gel silica 150 column (4.6 mm by 250 mm) (Tosoh) by means of established solvent systems (*9*, *32*). Eluent fractions were collected, and radioactivity was estimated by liquid scintillation counting (*22*). Authentic vitamin D derivatives [$1\alpha(OH)D_3$, $25(OH)D_3$, $24R,25(OH)_2D_3$, $1\alpha,25(OH)_2D_3$, and $1\alpha,24,25(OH)_3D_3$] were chromatographed, and the retention times of these vitamin D derivatives were determined by ultraviolet absorption at 264 nm [normal phase (**a**) and reverse phase (**d**)]. Reversed-phase HPLC (d to f) was run to confirm the presence of ^{3}H-labeled $1\alpha,25(OH)_2D_3$ with the use of a Cosmosil 5C18-AR packed column (4.6 mm by 150 mm) (Nacalai Tesque) (*32*). A_{264}, absorbance.

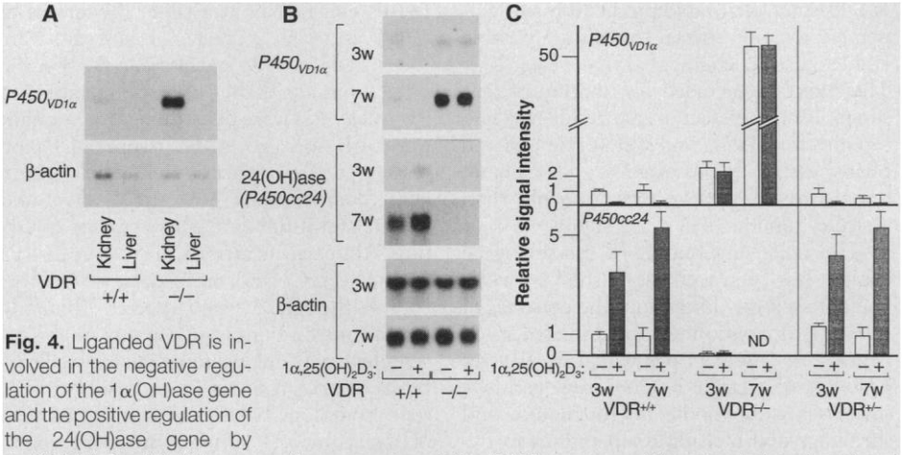

Fig. 4. Liganded VDR is involved in the negative regulation of the 1α(OH)ase gene and the positive regulation of the 24(OH)ase gene by $1\alpha,25(OH)_2D_3$. (**A**) Kidney-specific expression of the $P450_{VD1\alpha}$ gene. We extracted and analyzed poly(A)$^+$ RNA from various vitamin D target tissues, including kidney and liver, of the wild-type (+/+) and VDR-knockout (–/–) mice at 7 weeks by Northern (RNA) blot analysis using the $P450_{VD1\alpha}$ and the β-actin cDNA as probes (*26*). No transcript was detected in the other tissues, such as placenta (*21*). (**B** and **C**) Lack of response to $1\alpha,25(OH)_2D_3$ in the expression of the 1α(OH)ase and 24(OH)ase genes in the $VDR^{-/-}$ mice. Northern blot analysis of the $P450_{VD1\alpha}$ and 24(OH)ase (*P450cc24*) gene expressions was performed in the $VDR^{+/+}$, $VDR^{+/-}$, and $VDR^{-/-}$ mice at 3 and 7 weeks with (+) or without (–) an excess dose of $1\alpha,25(OH)_2D_3$ (50 ng per mouse) (*33*). A representative Northern blot analysis is shown in (B), and the relative abundance of the hydroxylase gene transcripts normalized with the β-actin transcript from more than five mice for one group is calculated (C). ND, not detected.

and $VDR^{+/-}$ mice, but not in the $VDR^{-/-}$ mice, at 3 and 7 weeks. The unusually high concentrations of serum $1\alpha,25(OH)_2D_3$ in the $VDR^{-/-}$ mice at 7 weeks (Fig. 1A) (*10*) are thus probably due to the overexpression of 1α(OH)ase. Thus, it is likely that liganded VDR inhibits 1α(OH)ase gene expression. In the $VDR^{-/-}$ mice, expression of 24(OH)ase, which converts $25(OH)D_3$ into $24R,25(OH)_2D_3$, was reduced to an undetectable amount, and the normal response (*24*) to $1\alpha,25(OH)_2D_3$ was not observed (Fig. 4, B and C). Thus, our results indicate that liganded VDR also regulates expression of 24(OH)ase, normally activating it (*25*).

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28. Polyadenylated [poly(A)$^+$] RNA was purified from the kidney of the VDR$^{-/-}$ mice at 7 weeks (*26*). The total cDNA was prepared with poly(A)$^+$ RNA (*27*). The cDNA fragments were subsequently inserted into the Hind III site of the mammalian expression vector pcDNA3 (Invitrogen). The reporter plasmid 17M2-G–*lacZ* was constructed by inserting yeast GAL4 upstream activating sequence x2 and β-globin promoter into a multicloning site of the Basic expression vector (Clontech). The ligand-binding domain of VDR [VDR(DEF)] fused to the GAL4 DNA-binding domain [GAL4-VDR(DEF)] (*11*) was used to detect ligand-induced AF-2 function. COS-1 cells cultured with Dulbecco's modified Eagle's medium containing 10% fetal bovine serum were transiently transfected with 0.5 μg of GAL4-VDR(DEF) expression vector, 1 μg of 17M2-G–*lacZ*, 0.2 μg each of the ADX and ADR expression vectors (*12*), and 0.1 μg of expression cDNA library in OPTI-MEM using lipofection agent (GIBCO BRL).
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30. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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32. The solvent systems were hexane/isopropanol/methanol (88:6:6) as the mobile phase at a flow rate of 1.0 ml/min for normal phase and methanol/H_2O (80:20) at a flow rate of 1.0 ml/min for reverse phase. On both normal- and reversed-phase HPLC with other solvent systems [hexane/isopropanol (90:10), hexane/isopropanol (80:20) for normal phase, and methanol/H_2O (75:25) for reversed phase] (*22*), the retention times of the enzymatic product also completely matched those of authentic 1α,25$(OH)_2D_3$ (*21*).
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Extensible Collagen in Mussel Byssus: A Natural Block Copolymer

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To adhere to solid surfaces, marine mussels produce byssal threads, each of which is a stiff tether at one end and a shock absorber with 160 percent extensibility at the other end. The elastic extensibility of proximal byssus is extraordinary given its construction of collagen and the limited extension (less than 10 percent) of most collagenous materials. From the complementary DNA, we deduced that the primary structure of a collagenous protein (preCol-P) predominating in the extensible proximal portion of the threads encodes an unprecedented natural block copolymer with three major domain types: a central collagen domain, flanking elastic domains, and histidine-rich terminal domains. The elastic domains have sequence motifs that strongly resemble those of elastin and the amorphous glycine-rich regions of spider silk fibroins. Byssal thread extensibility may be imparted by the elastic domains of preCol-P.

Mussel byssal threads are undoubtedly among nature's most peculiar tendons. One end of each thread inserts into the byssal retractor muscles at the base of the foot; the other end is disposed outside the animal and attached to a hard surface by an adhesive plaque. In a process that resembles injection molding, the foot of the mussel is able to make a new thread in 5 min or less (*1*). Despite their rapid production and vulnerable location, byssal threads are durable and exquisitely engineered fibers (Fig. 1A). They contain a graded distribution of tensile molecular elements that result in a material that is strong and stiff at one end and pliably elastic at the other (*2*, *3*). Overall, byssal threads are five times tougher than Achilles tendon (*4*).

The collagen content of mussel byssal threads has been well established by wide-angle fiber x-ray diffraction, the presence of *trans*-4-hydroxyproline, and byssus-derived pepsin-resistant peptides with Gly-X-Y repeats (*5*, *6*). Unlike tendon, however, byssus has a nonperiodic microstructure and shrinkage and melting temperatures in excess of 90°C (*5*). Any attempt to reconcile these unusual biochemical and mechanical features with what is known about collagen provokes the following molecular questions: How is byssal collagen different from tendon collagen, and what role does this difference play in the material performance of byssus?

Because of its highly cross-linked structure, there has been only one recourse for recovering collagen from byssus: acid extraction coupled with extensive treatment with pepsin. We (*6*) used this approach to characterize two collagen fragments, Col-P and Col-D (both apparently homotrimers), with apparent α-chain masses of 50 and 60 kD, respectively (*7*). These fragments coexist in complementary gradients along the length of each byssal thread with Col-D predominating at the distal end and Col-P predominating at the proximal end. Similar gradients were found to exist in the mussel foot, which fabricates the byssus one thread at a time. Precursors to Col-P and Col-D (preCol-P and -D) were identified in foot extracts with specific polyclonal antibodies. Apparent masses of 95 and 97 kD were determined for α-chain preCol-P and preCol-D (α-preCol-P and α-preCol-D), respectively. The amino acid compositions for the NH_2- and COOH-terminal extensions consist of Gly plus Ala concentrations of 50 (preCol-P) and 60 (preCol-D) mole percent (mol%) (*6*). Although it was inferred that the extensions or flanking domains might resemble structural proteins such as silk fibroin or elastin or resilin, confirmation of such homology has awaited determination of the complete primary structure. Here we report the primary structure of α-preCol-P, a natural block copolymer with putative cross-linking, elastic, and collagen domains (*8*).

α-PreCol-P may be divided into seven domains based on its amino acid sequence (Fig. 2). The most prominent feature is a central region consisting of a 435-residue collagenous sequence identified by alignment with amino acid sequences reported previously for peptides derived from the pepsin-resistant fragment Col-P (*6*). The collagen domain consists of 146 Gly-X-Y repeats in which X and Y are frequently Pro. Only the Pro residues at position Y appear to be converted to *trans*-4-hydroxyproline (*6*). Gly also occurs in the X (10 times) and Y (once) positions. The continuity of Gly-X-Y repeats is interrupt-

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