Structure/Function Relationships in the Minicollagen of Hydra Nematocysts*

Received for publication, September 13, 2002, and in revised form, October 2, 2002
Published, JBC Papers in Press, October 3, 2002, DOI 10.1074/jbc.M209401200

Suat Özbek‡, Olivier Pertz§, Martine Schwager‡, Ariel Lustig‡, Thomas Holstein¶, and Jürgen Engel††

From the ‡Department of Biophysical Chemistry, Biozentrum, University of Basel, Klingelbergstrasse 70, CH-4056 Basel, Switzerland, §Department of Cell Biology, The Scripps Research Institute, La Jolla, California 92037, and Institute of Zoology, Technical University of Darmstadt, Schnittbachstrasse 10, D-64287 Darmstadt, Germany

The minicollagens found in the inner layer of the Hydra nematocyst walls are the smallest collagens known with 12-16 Gly-X-Y repeats. Minicollagen-1, the best characterized member of this protein family so far, consists of a central collagen triple helix of 12 nm in length flanked at both ends by a polyproline stretch and a conserved cysteine-rich domain. The cysteine-rich tails are proposed to function in the assembly of soluble minicollagen trimers to high molecular structures by a switch of the disulfide linkage from intramolecular to intermolecular bonds. In this study, we investigate the trimeric nature of minicollagen-1 and its capacity to form disulfide-linked polymers in vitro. A fusion protein of minicollagen-1 with maltose-binding protein is secreted as a soluble trimer with only intrachain and no interchain disulfide bridges as confirmed by melting the collagen triple helix under reducing and non-reducing conditions. The conversion of minicollagen-1 trimers to monomers takes place between 40 and 55 °C with the melting point being ~45 °C. Oxidative reshuffling of the minicollagen-1 trimers leads to the formation of high molecular aggregates, which upon reduction show distinct polytrimeric states. Minicollagen trimers in isolated nematocyst capsules proved to be sensitive to SDS and were engaged in polymeric structures with additional cross-links that were resistant to reducing agent.

Nematocysts are explosive organelles of defense, capture of prey, and locomotion found exclusively in Cnidaria (Coelenterates) like Hydra, jellyfish, and corals. A specialized cell type, the nematocyte, produces them during a complex cellular secretion process that involves the formation of the capsule and its long cylindrical tube from a giant post-Golgi vesicle by a regulated assembly of different protein layers (1, 2). The final maturation step of the cyst is characterized by a compaction and hardening of the capsule wall, during which the matrix of the capsule is filled with poly-y-glutamate (2 m) and its cations, leading to an increase of the internal osmotic pressure to 150 megapascals (3, 4). Upon triggering, the capsule is discharged and ejects the inverted tube in an ultra-fast (<3 ms) exocytosis process driven by the kinetic energy stored in the capsule wall (5).

The existence of hydroxypoline-rich collagens in the wall and tubule structures of nematocysts was suggested very early by amino acid analysis of Hydra nematocyst proteins (6). Further experiments revealed that the collagenous proteins contained in the nematocysts obviously formed disulfide-linked polymers soluble only under reducing conditions (7). A first step in unraveling the molecular basis of the nematocyst wall was taken in isolating a family of genes coding for short collagen-like molecules from a Hydra cDNA library (8). The Hydra minicollagens up to now comprise seven members with a characteristic modular architecture that is also reflected in a related transcript isolated from a reef-building coral, Acropora donei (9). The central collagen (Gly-X-Y) region is flanked on both sides by a probably hydroxylated polyproline stretch followed by a cysteine-rich domain that is highly conserved among some of the minicollagen molecules including minicollagen-1. During nematocyst morphogenesis, minicollagens are assembled to the electron-lucent inner layer of the capsule wall that is covered on the outside by densely packed globular particles containing the NOWA1 protein (8, 10, 11). Atomic force microscopy and biochemical evidence showed that minicollagens form a dense fibrillar structure in the inner wall accessible only by reducing agents in mature nematocysts (10, 12).

We were able to express minicollagen-1 in a eucaryotic expression system using 293 cells (12). A fusion protein containing a C-terminal His-tag and the native signal and prosequences was properly processed and secreted but showed a high tendency for self-aggregation. To confirm the trimeric state of minicollagen-1, a fusion protein with maltose-binding protein (MBP) linked to the C terminus, was designed and expressed in 293 cells. Minicollagen-1-MBP proved to be soluble under native conditions and showed trimers of the globular MBP parts on transmission electron micrographs.

Here we characterize the trimeric nature of the minicollagen-1-MBP fusion construct and its capacity to form polymeric networks triggered by an in vitro reshuffling process. Minicollagen detected in isolated capsules formed insoluble polymers in mature wall structures that even after reduction showed polymeric fractions suggesting a different type of cross-linking.

EXPERIMENTAL PROCEDURES

cDNA Constructs and Transfection Procedures—The minicollagen-1-MBP fusion construct was designed as described previously (12). MBP cDNA was fused to the C terminus of minicollagen-1 using the overlap extension method (22), and the resulting fragment was subcloned into the pCEP-Pu vector for expression in mammalian cells. For stable transfection, 293 EBNA cells were kept in Dulbecco’s modified Eagle’s medium F12 supplemented with 10% fetal bovine serum, 1% Gln, and Pen/Strep. Cells were grown to 80% confluence in 6-well plates and

¶ The abbreviations used are: NOWA, nematocyst outer wall antigen; MBP, maltose-binding protein.

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
transfected overnight with 1 μg of vector DNA using 5 μl of LipofectAMINE reagent. The selection of positive clones was performed by culturing transfected cells with 2 μg/ml puromycin with frequent changes of medium until a resistant population appeared. All reagents were purchased from Invitrogen.

Expression and Purification—EBNA 293 cells stably transfected with minicollagen-1-MBP cDNA were grown to high density in 125-ml cell culture flasks using complete medium. For expression of recombinant fusion protein, cells were switched to serum-free expression medium containing 500 μg/ml ascorbic acid to ensure hydroxylation of the prolines within the collagen sequence (13). Cell supernatants were harvested frequently until the cells detached, pooled, and passed through a syringe filter. Conditioned medium was then purified using amylose affinity chromatography according to the manufacturer’s instructions (New England Biolabs). Eluted fractions were analyzed by SDS-PAGE and dialyzed against 50 mM Tris-HCl, pH 8.0, and 150 mM NaCl.

SDS-PAGE and Western Blot Analysis—Before solubilizing in non-reducing sample buffer, samples were incubated with different concentrations of β-mercaptoethanol as indicated in the experiments. Samples were not boiled to preserve the trimeric state of the minicollagen molecules. The melting of the triple helix was performed by incubating samples for 10 min in a water bath (Julabo) at increasing temperatures and treated immediately with sample buffer to prevent refolding. For resolving minicollagen-trimers and higher aggregates, non-reducing gradient gels (3–10%) were applied with 2.5% stacker gels to ensure that high molecular complexes entered the separating gel. Protein bands were visualized by silver staining. Western blot analysis was performed using minicollagen polyclonal antibody (rabbit) generated against His-minicollagen-1 (12). Primary antibody (1:1000) was detected using an anti-rabbit-horseradish peroxidase conjugate antibody (1:2000) and the ECL chemiluminescence system (Amersham Biosciences).

Analytical Ultracentrifugation—A Beckman model XLA analytical ultracentrifuge equipped with absorption optics was employed. Sedimentation velocity runs were performed at 54000 rpm and sedimentation equilibrium runs were performed at 16000 rpm at a filling height of 0.5 cm. All measurements were performed at 20 °C. The molecular masses were calculated from sedimentation equilibrium runs using a floating base-line computer program that adjusts the base-line absorption to obtain the best linear fit of ln A versus r² (A = absorbance; r = distance from the rotor axis). A partial specific volume of 0.73 cm³/g for minicollagen-1-MBP was used for the calculation, and the sedimentation velocity coefficient was corrected to standard conditions (H₂O at 20 °C).

Preparation of Minicollagen from Nematocyst Walls—Intact undischarged nematocysts were isolated from whole Hydra tissue as described previously (14). Approximately 1 × 10⁶ capsules were suspended in 500 μl of 50 mM Tris-HCl, pH 7.4, and 150 mM NaCl and sonicated for 1 h at room temperature using a water bath (Elgasonic). Capsules were centrifuged for 5 min at 6000 rpm in a table centrifuge (Heraeus), and the supernatant was carefully removed. Capsule supernatants were then taken for Western blot analysis using different concentrations of β-mercaptoethanol. Samples were not heated to preserve minicollagen-trimers in the preparations.

RESULTS

The minicollagen-1-MBP fusion protein was designed as described with maltose-binding protein added to the C terminus via a flexible linker of 12 amino acids (Fig. 1A) (12). The MBP fusion part enabled solubility and purification of the minicollagen-1 molecules under native conditions. As MBP does not contain cysteines, it is supposed not to interfere with the disulfide connectivity and oligomeric state of the minicollagen molecules. Minicollagen-1-MBP was expressed and secreted by 293 cells exclusively as a soluble trimer with an apparent molecular mass of ~190 kDa (Fig. 1B). The trimer was resolved with the use of a non-reducing polyacrylamide gradient (3–10%) gel charged with unheated samples to prevent denaturation of the triple helix. The calculated mass for the minicollagen-1-MBP monomer is 58 kDa, which corresponds well to the apparent molecular mass on SDS gels (60 kDa). As noted earlier, upon incubation of minicollagen samples with reducing agent the apparent molecular mass slightly shifted up probably because of a more extended shape of the minicollagen-1 molecules caused by the dissociation of intrachain disulfide bonds.

The trimeric state of minicollagen-1-MBP was additionally confirmed by analytical ultracentrifugation (Table I). Minicollagen-1-MBP sedimented with a single profile and a sedimentation coefficient of 5.7 S. Sedimentation equilibrium yielded a molecular mass of 168 kDa, which corresponds to a trimerized fusion molecule. The frictional ratio f/f₀ = 1.6 suggests an elongated shape of the trimeric protein reflecting the rodlike appearance of minicollagen-1 molecules observed in scanning transmission electron microscopy (12).

We next asked whether interchain disulfide bonds were involved in the formation of the collagen triple helix or whether all cysteinyl residues were engaged in intrachain bridges only. Minicollagen-1-MBP samples were treated with increasing concentrations of β-mercaptoethanol followed by incubation at room temperature or 100 °C for 10 min and analyzed using
conditions using minicollagen-1 antibody for detection. As shown in Fig. 2, non-heated samples displayed the observed molecular shift with reduction while completely retaining the trimeric state (left panel). The heating of minicollagen-1-MBP, however, lead to quantitative denaturation of the collagen triple helix in the presence and absence of reducing agent (right panel). Upon the reduction of the boiled samples, a pronounced double band could be observed that probably indicates heterogeneous hydroxylation of the prolines within the collagen and polyproline stretches. These data demonstrate that the cysteine-rich tails are not involved in the maintenance of the trimeric minicollagen structure but instead form independent domains with initially all-intrachain disulfide bonds.

To estimate the thermal stability of the collagen triple helix, minicollagen-1-MBP samples were incubated at rising temperatures and the dissociation of the triple helix was assayed by gradient SDS-PAGE (Fig. 3). Melting took place between 35 and 60 °C. The Tm value for the triple helix under reducing conditions (1 mM β-mercaptoethanol) was ~45 °C (Fig. 3, left panel). The same experiment performed under non-reducing conditions (Fig. 3, right panel) did not show any alteration of the melting point, confirming that there is no contribution to trimer stability by interchain disulfide bonds within a trimer.

The structural model for the minicollagens in the nematocyst wall (10) suggests that they form disulfide-linked polymers in which the N- and C-terminal domains overlap, yielding a repeating pattern of collagen helix and cysteine-rich domains. To reproduce this polymerization process in vitro, we reduced minicollagen-1-MBP quantitatively by treatment with 10 mM dithiothreitol and allowed it to reoxidize slowly by dialysis against non-reducing buffer. An analysis of the reoxidized samples by gradient SDS-PAGE revealed that high molecular aggregates had been formed that hardly entered the separating gel under non-reducing conditions (Fig. 4A). Upon reduction, these polymers were gradually dissolved displaying a ladder of protein bands with molecular weight sizes matching polymeric minicollagen-1-MBP trimers. Nearly a total reduction to trimers was achieved by applying β-mercaptoethanol concentrations above 1 mM. We did not observe any precipitation as a result of the polymerization process, which might be explained by the limiting protein concentration (0.2 μM) applied in these experiments. To confirm that reoxidation led to disulfide-linked polymers only, SDS-PAGE was performed with boiled samples (Fig. 4B), demonstrating that minicollagen-1-MBP polymers are resistant to heat and dissociated to monomers only upon reduction.

We have shown earlier that minicollagen-1 antibody recognizes distinct antigens in mature nematocysts, displaying several bands between 30–50 kDa that are attributed to different members of the minicollagen family (12). To investigate the polymeric state of the minicollagens in nematocysts, we isolated wall proteins under non-reducing conditions by mild sonication of purified capsules in a water bath. This treatment led to partial dissociation of the outer wall structure while retaining capsule integrity as confirmed by light microscopy. The sonicated capsules were centrifuged, and the supernatant was taken for Western blotting under reducing and non-reducing conditions using minicollagen-1 antibody for detection. As shown in Fig. 5A, no minicollagen proteins could be detected in the non-reduced supernatant, showing that the total minicollagen fraction was engaged in high molecular weight aggregates. Incubation with increasing concentrations of reducing agent allowed the separation of four minicollagen species ranging from 36 to 70 kDa with the higher bands being overrepresented, but most of the material remained in polymeric state not penetrating the separating gel. The 70-kDa band was not observed in capsule samples before (12) and might represent a cross-reaction with a longer member of the minicollagen family. A second fraction of bands could be detected in the molecular mass range between 100 and 120 kDa, and by treatment of the capsule supernatant with 1 mM β-mercaptoethanol, higher aggregates were made visible that were diffused by glycosylation. Heating of the samples did not result in a qualitative change of the band pattern, demonstrating that collagen trimers in the capsule wall were obviously SDS-sensitive already at low temperatures. The resistance of the polymeric fractions to reducing agents observed in this experiment points to a further mechanism of intermolecular connectivity beside that facilitated by disulfide bonds.

In a second experiment, purified capsules were applied to Western blotting under reducing and non-reducing conditions (Fig. 5B). In contrast to the minicollagen fractions of mature cell wall fragments, capsules contained yet unpolymerized moieties running at 70 and 100 kDa, respectively (left lane in both panels). Reduction by β-mercaptoethanol produced the same pattern of monomer and polymer fractions as in Fig. 5A, but most of the capsule material remained insoluble and did not enter the separating gel. Nearly all oligomeric and polymeric fractions could be converted to monomers by heat denaturation (right panel). Non-reduced polymers were heat-stable in SDS similar to the polymers of the recombinant minicollagen-1-MBP.

**TABLE I**

| Minicollagen-1-MBP | Concentration (μg/ml) | Molecular mass (kDa) | Sedimentation coefficient (Ss, 40°C) |
|-------------------|----------------------|---------------------|----------------------------------|
| Trimer            | 200                  | 168                 | 174                              |

**FIG. 2. Analysis of intrachain disulfide bonds.** Minicollagen-1-MBP samples were incubated for 5 min using different concentrations of β-mercaptoethanol as indicated. Samples shown on the right panel were boiled subsequently for 10 min.

As shown in Table I, the minicollagen-1-MBP polymers were engaged in high molecular weight aggregates. The resistance of the polymeric fractions to reducing agents observed in this experiment points to a further mechanism of intermolecular connectivity beside that facilitated by disulfide bonds.

**FIG. 3.** Assembly of Hydra Minicollagen

**FIG. 4.** Analysis of intrachain disulfide bonds in capsules after treatment with dithiothreitol and subsequent reoxidation with β-mercaptoethanol at different concentrations.

**FIG. 5.** Western blotting of minicollagen-1-MBP under reducing and non-reducing conditions.
strength has been estimated to be near that of steel (10). On the other hand, the volumetric changes during a nematocyst discharge suggest some flexibility, allowing the storage of mechanical energy in the wall structure. It was proposed that to meet these requirements, the minicollagen trimers are arranged linearly to form disulfide-linked protofilaments spread around the capsule wall in different layers with shifted angles. The fine structure of a filament analyzed by atomic force microscopy revealed a repetitive pattern consistent with the periodicity of triple helix and polyproline stretches of the linearly assembled minicollagens.

Fig. 3. Melting of the minicollagen triple helix as demonstrated by SDS-PAGE. Minicollagen-1-MBP samples were incubated for 10 min in the presence (left panel) or absence (right panel) of 1 mM β-mercaptoethanol at different temperatures. Non-reducing sample buffer was added immediately after incubation to prevent refolding of the collagen trimer.

Fig. 4. A, analysis of interchain disulfide bonds. Minicollagen-1-MBP was reduced using 10 mM dithiothreitol and allowed to reoxidize by dialysis. Samples were treated with increasing concentrations of β-mercaptoethanol for 5 min as indicated and analyzed by gradient (3–10%) SDS-PAGE without heating. B, same sample treated with increasing concentrations of β-mercaptoethanol with subsequent boiling.

Fig. 5. A, nematocyst capsules were isolated from Hydra magnipapillata by elutriation and sonicated in a water bath for 1 h. Soluble wall fragments in the capsule supernatant were submitted to Western blotting with minicollagen-1 antibody. Samples were treated with β-mercaptoethanol concentrations as indicated without subsequent boiling. B, Western blot of nematocyst capsules treated with different concentrations of β-mercaptoethanol as indicated with (right panel) and without (left panel) boiling. Lanes with higher concentrations of reducing agent were omitted because of intense signals.
Our results provide the first biochemical evidence for triple helix formation of a recombinant minicollagen molecule from *Hydra* and its capacity to form disulfide-linked polymers similar to those found in mature capsule walls. Minicollagen-1-MBP was expressed in a mammalian system ensuring proper folding of the cysteine-rich domains and hydroxylation of prolines in Y positions within the collagen part because of the presence of prolyl-4-hydroxylase. SDS-PAGE showed that purified minicollagen-1-MBP existed exclusively as a trimer reflecting the soluble precursor form of minicollagens in immature nematocytes. As observed earlier for His$_6$-minicollagen-1, the reduction of minicollagen-1-MBP led to an increase of the apparent molecular weight of 5–10%, indicating a more compact shape of the cysteine-rich domains in the non-reduced state. Trimerization was also confirmed by ultracentrifugation analysis, which revealed a fractional ratio of 1.6, suggesting a distinctly non-globular shape for the full-length protein. This finding is conform with the scanning transmission electron microscopy analysis of His$_6$-minicollagen-1, which had shown rod-like particles with a central stalk of ~13 nm in length and 4 nm in diameter from which the polyproline helices protruded as shorter rods of ~7 nm (12).

The transition temperatures of collagens from different sources have been shown to be quite close to the upper limit of the physiological temperatures of the donor species (15). The measured heat stability of 45 °C in our study is 9 °C above the temperature tolerance described for *Hydra viridissima* (35–36 °C), the difference being even more pronounced if compared with the temperature tolerance of stenothermal species (24–25 °C) (16). The melting of the minicollagen-1 triple helix under reducing conditions did not decrease the transition point as described recently for a “minicollagen” in which the three chains containing the COL1 and NC1 domains of collagen XII are disulfide-linked (17). This confirms that the cysteine-rich domains of minicollagen-1 do not function in triple helix stabilization, indicating that the high stability of minicollagen-1 conferred mainly by the hydroxylation of prolines in the Y positions, is supposed to increase the heat stability as a consequence of an inductive effect.

Total reduction and reoxidation of the purified minicollagen-1-MBP resulted in the formation of high molecular polymers that were resistant to SDS at 100 °C but could be reduced to monomeric and polytrimeric bands by reopening of disulfide bonds (Fig. 4, A and B). The same result was obtained when the reshuffling was induced enzymatically by incubation with protein disulphide isomerase in the presence of 1 mM dithiothreitol or by applying a glutathione reoxidation system (data not shown). Minicollagen-1-MBP did not show any tendency to aggregate in non-reduced state as confirmed by ultracentrifugation experiments performed with protein concentrations as high as 10 μM. We conclude from this that partial reduction is sufficient to trigger the polymerization of minicollagens, which might be an enzymatically controlled process in the nematocyst morphogenesis probably induced by changes in the redox state of the matrix. A similar disulfide exchange process was described for the formation of von Willebrand factor multimers in promoting platelet adhesion (18). Very recently, crystallographic evidence provided a molecular mechanism for the cystine-dependent dimerization of prion protein PrP, which involved domain swapping (19). Such a symmetric rearrangement would need only a minimum of free energy as the new linkage is made with an identical structural unit provided by the partner molecule. We speculate that a similar dimerization process might hold true for the cysteine-rich domains of the *Hydra* minicollagens (20). This mechanism of polymerization would only require the opening of a single exposed cysteine bridge while retaining the overall structure of the domain. Minicollagen in isolated nematocytes as detected by the minicollagen-1 antibody showed different properties from the recombinant minicollagen-1-MBP, probably because of different post-translational modifications during the process of capsule formation. Most of the minicollagen was engaged in aggregates that could be solubilized only partially by applying reducing agents. Polymorphic structures from mature wall fragments isolated by mechanical force in SDS gels displayed minicollagen fractions only after reduction, whereas capsules contained also soluble minicollagen molecules. Trimeric molecules were sensitive to SDS, and reduction led to dissociation to monomers already at room temperature. The partially solubilized polymorphic fractions, in contrast to those of recombinant minicollagen-1-MBP, proved resistant to high concentrations of reducing agent, indicating that minicollagen molecules in nematocytes were interlinked not only by disulfide linkage but also by other covalent cross-links that might be essential for the correct assembly of the capsule wall. Such intermolecular cross-links have been described to be of the same nature in higher vertebrates as well as in primitive invertebrates (21).

Nematocyst morphogenesis follows a very complex developmental pathway that involves the gradual deposition of proteins on the inner side of a post-Golgi vacuole to form the capsule wall. We have shown by immunolocalization that the assembly of minicollagen-1 in this process is preceded by the appearance of the NOWA protein in *Hydra* (11). NOWA forms the outer layer of the nematocyst capsule and is believed to play a regulatory role in minicollagen assembly as its C-terminal part consists of an octad repeat of a cysteine-rich domain similar to that in minicollagens. Therefore, it was suggested that NOWA might form covalent links with minicollagen molecules via disulfide bonding (11). Our experiments show that by mechanical removal of soluble outer wall particles, it is possible to isolate minicollagen that is exclusively engaged in high molecular aggregates. These complexes proved to be sensitive to reduction as the polymers formed by the recombinant minicollagen-1-MBP after reoxidation. It remains to be analyzed whether these aggregates are composed of minicollagen only or represent heterogeneous complexes with NOWA protein.

**REFERENCES**

1. Skaer, R. J. (1973) *J. Cell Sci.* 13, 371–393
2. Holstein, T. (1981) *J. Ultrastruct. Res.* 75, 276–290
3. Weber, J., Schurthold, T., and Neumann, E. (1990) *Trends* 28, 403–409
4. Szczepanke, S., Cikala, M., and David, C. N. (2002) *J. Cell Biol.* 155, 745–751
5. Holstein, T., and Tartdert, P. (1984) *Science* 223, 830–833
6. Lenhoff, H. M., Kline, E. S., and Hurley, R. (1985) *Biochem. Biophys. Acta* 204, 204–205
7. Blanquet, R., and Lenhoff, H. M. (1966) *Science* 154, 152–153
8. Kura, E. M., Holstein, T. W., Petri, B. M., Engel, J., and David, C. N. (1991) *J. Cell Biol.* 115, 1159–1169
9. Anderhub, G., Podlesek, Z., and Macke, P. (2000) *Biochem. Biophys. Acta* 1476, 372–376
10. Holstein, T. W., Benoît, G. M., Herder, G. v., Wanner, G., David, C. N., and Gaub, H. E. (1994) *Science* 265, 402–404
11. Engel, U., Orbek, S., Engel, R., Petri, B., Löttspeich, F., and Holstein, T. W. (2003) *J. Cell Biol.* 155, 293–304
12. Engel, U., Pertz, O., Fauser, C., Engel, J., David, C. N., and Holstein, T. W. (2001) *EMBO* 20, 3063–3073
13. Myllyharju, J., Nakelainen, M., Vuorela, A., and Kivirikko, K. I. (2000) *Biochem. Soc. Trans.* 28, 353–357
14. Weber, J. (1990) *J. Biol. Chem.* 265, 9664–9669
15. Rigby, R. J., and Robinson, M. S. (1975) *Nature* 253, 277–279
16. Schroeder, L. A., and Callaghan, P. S. (1981) *Limnol. Oceanogr.* 24, 1092–1103
17. Mazzorana, M., Cogne, S., Goldschmidt, D., and Auveert-Foucher, E. (2001) *J. Biol. Chem.* 276, 2793–2794
18. Wagner, D. G., Lawrence, S. O., Holzmann-Wilhelm, B. M., Fay, P. J., and Marier, V. J. (1987) *Blood* 69, 27–32
19. Knapp, K. J., Morillas, M., Swienton, W., Malone, M., Surewicz, W. K., and Yee, V. C. (2001) *Nat. Struct. Biol.* 8, 770–774
20. Orbek, S., Engel, U., and Engel, J. (2002) *J. Struct. Biol.* 137, 11–14
21. Bailey, A. J. (1971) *PERS Lett.* 19, 154–158
22. Ho, S. (1990) *DNA Protein Eng.* 2, 50–55
23. Engel, J. (1997) *Science* 277, 1785–1786