ACAA, but this represents only a 0.7-kcal/mol decrease in ΔG° of folding at 70°C. An equal or larger gain in ΔG° could be achieved by changing any AU base pair in a stem to GC (6). With the ΔG°s of Freier *et al.* (6) for base pairs and the approximation of a terminal GA mismatch as AG (*12*), the hairpin loops of CAAAAG and CUUUUG in 1 M NaCl studied by Groebe and Uhlenbeck (*20*) have loop free energies of 4.2 and 3.9 kcal/mol at 70°C, slightly more stable than the 5.1 kcal/mol for the GCAA loop in 0.1 M NaCl studied here. Thermodynamic parameters measured by Antao *et al.* (*21*) show that a CGAAAG hairpin loop is only 0.3, 0.5, and 0.2 kcal/mol more stable at 70°C than CGCUUG, CUUUUG, and CUUUGG loops, respectively. The phylogenetic preference for GNRA tetraloops (9) is probably due to nonthermodynamic factors, such as the ability to form tertiary contacts (*22*). Woese *et al.* (9) show that given tetraloops are replaced specifically rather than randomly by other tetraloops. For example, 93% of the hairpin loops at position 83 in 16S rRNA have the sequence CUUG, UUCG, or GCAA (9). NMR studies indicate both UUCG and GCAA loops have defined structures (*8, 16*). Thus, an alternative explanation for the context dependence of hydrogen bonding is that the ΔG° contribution may help to hold the loop in a single conformation.

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18. Two-dimensional NMR experiments (*19*) for the duplex $(rGGCGAGCC)_2$ are consistent with the G to A hydrogen bonding shown in Fig. 3. The duplex $(rGGCIAGCC)_2$, however, is more stable than $(rGGCGAGCC)_2$ (*12*), which is surprising for the hydrogen bonding in Fig. 3. NMR spectra of $(GGCIAGCC)_2$, however, show a resonance at 14.3 ppm for the imino proton of I (*19*), which indicates the hydrogen bonding in the IA mismatch involves the imino proton. Thus the increase in stability for $(rGGCIAGCC)_2$ as compared to $(GGCGAGCC)_2$ is associated with a change in structure.
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Centriole Duplication in Lysates of *Spisula solidissima* Oocytes

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A cell-free system has been developed that executes centriole duplication. Surf clam (*Spisula solidissima*) oocytes, arrested at late prophase of meiosis I, do not contain centrioles, centrosomes, or asters. Serial section high-voltage electron microscopy (HVEM) of asters and spindles isolated from potassium chloride–activated oocytes indicates that within 4 minutes oocytes assemble a single centriole that is duplicated by 15 minutes when assembly of the first meiotic spindle is complete. A mixture of lysates from unactivated oocytes and potassium chloride–activated oocytes induces centriole formation and duplication. Astral microtubule content in these lysate mixtures increases with time.

Centrioles are structurally complex cylindrical organelles associated with centrosomes, asters, cilia, and flagella of animal cells (*1, 2*). Within the centrosome, centrioles are commonly found as a mother and daughter pair intimately associated with ancillary pericentriolar structures. During interphase these pericentriolar structures serve as nucleation sites for cytoplasmic microtubules (MTs), whereas during mitosis they generate radial astral arrays of MTs that form the spindle (*3, 4*). The initiation of centrosome replication, which is a preparatory step for mitosis, is signaled by centriole replication; in echinoderm embryos the ability of a centrosome to replicate depends on the presence of a preexisting centriole (*5, 6*). The controlled replication of centrioles in sea urchin (*7, 8*) and frog zygotes (9) and their subsequent separation to form functionally independent centrosomes occurs in the absence of both mRNA and protein synthesis. Thus, in these developing embryos new centrioles and functional centrosomes are assembled within the zygote from preexisting pools of subunits. Unfortunately, centrioles and their associated structures have yet to be isolated in purified form, and their composition and replication are poorly understood (*10–12*). Here we describe a cell-free system based on *Spisula solidissima* oocytes that supports centriole assembly and duplication.

When *Spisula* oocytes are arrested at late prophase of meiosis I, centrosomes cannot be detected in living oocytes by high-resolution light microscopy (*13, 14*) or in fixed oocytes by electron microscopy (EM) (*16, 17*) or tubulin immunofluorescence (IMF) (*15*). Moreover, lysates from unactivated oocytes do not contain or form asters (*18*). When oocytes are parthenogenetically activated with KCl they complete meiosis I and II (*13, 14*), and within 2 to 5 min of activation two astral arrays of MTs can be detected in the cytoplasm near the germinal vesicle (*15*). These asters are also found in lysates prepared from eggs 4 min after activation (Figs. 1 and 2) and appear to contain a single centriole when examined in random thin sections (*18, 19*). Indeed, high-voltage EM (HVEM) analyses (*20*) of serial thick sections through ten of these asters confirmed that each contains only a single centriole (Fig. 2).

Approximately 15 min after activation oocytes contain a mature meiosis I metaphase spindle (*13–17*). All ten centrosomes examined by serial-section HVEM that

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were associated with meiosis I spindles isolated from *Spisula* oocytes (*20*) contained two centrioles (Fig. 2). In some cases these two centrioles were adjacent to each other and orthogonally arranged, whereas in other instances they showed no preferred orientation and were separated by the thickness (≥0.25 μm) of one or more sections. We conclude that unactivated *Spisula* oocytes contain no functional centrosomes but upon activation with KCl rapidly assemble two functional centrosomes, each of which contains a single centriole (*15–19*). The centrioles within these centrosomes then duplicate by the time of meiosis I metaphase, approximately 15 min after activation.

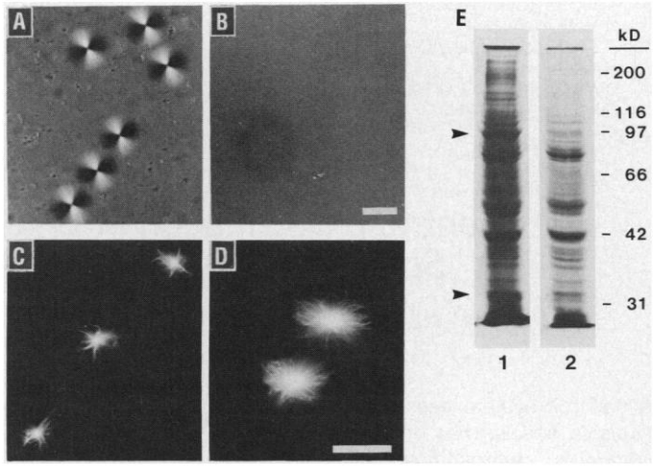

Fig. 1. Polarized light and tubulin IMF micrographs of *Spisula* asters. (**A**) Polarized light micrograph of asters prepared as described (*19, 20*) from lysates of oocytes 4 min after activation. (**B**) Polarized light micrograph of lysate after asters were removed by high-speed centrifugation (HSA-extract) (*21*). (**C**) Tubulin IMF micrograph of numerous small asters present within 4 min of incubation with equal volume mixture of HSA-extract and U-lysate (*21*). (**D**) Tubulin IMF micrograph of asters in (C) after 15 min. (**E**) Comparison of proteins by SDS–polyacrylamide gel electrophoresis (*20, 22*). Proteins present in A/4-lysates (lane 1) were depleted from HSA-extracts (lane 2) by centrifugation. Prominent proteins of approximately 33 and 97 kD (arrowheads) were removed. Bars in (B) and (D), 20 μm; (A) and (B), same magnification; (C) and (D), same magnification.

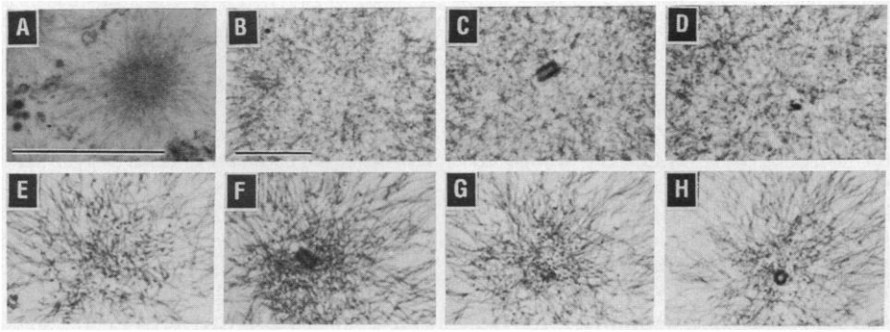

Fig. 2. High-voltage electron micrographs of serial 0.25-μm-thick sections through asters from activated *Spisula* oocytes (*20*). (**A**) Asters in lysates of oocytes prepared 4 min after activation. (**B** through **D**) Three serial sections of an aster in (A). A single centriole is visible in (C). Four serial sections through an aster (**E** through **H**) associated with the metaphase spindle of meiosis I and isolated 15 min after activation reveal two centrioles (F and H) separated by one serial section (G). Bar in (A), 10.0 μm; bar in (B), 1.0 μm; (B through H), same magnification.

To determine cell-free conditions for centriole duplication, we prepared lysates from *Spisula* oocytes 4 min after activation (A/4-lysates). We depleted these A/4-lysates of their asters and their ability to spontaneously form asters by centrifugation (Fig. 1B), generating cleared lysates (HSA-extracts) (*21*). Comparison by gel electrophoresis of proteins in lysates and HSA-extracts (Fig. 1E) revealed that numerous proteins were removed by centrifugation but did not show which of these proteins were components of asters, centrioles, or centrosomes. We also prepared lysates from unactivated oocytes (U-lysates) (*21*); these lysates did not contain or spontaneously form asters (*18*).

Equal volumes of HSA-extract and U-lysate were then combined and warmed to room temperature. At various times aliquots were removed from the mixture and prepared for analysis by either tubulin IMF or HVEM (*21*). IMF revealed that by 4 min the mixtures contained asters (Fig. 1C) and that by 15 min the asters had increased in diameter and fluorescence (Fig. 1D). An extensive HVEM analysis of serial thick sections through 15 asters, obtained after a 4-min incubation of the lysate mixture, revealed that each aster contained a single centriole (Fig. 3, A through E). By comparison, all 22 asters reconstructed by HVEM after a 15-min incubation of the lysate mixture contained two centrioles each (Fig. 3, F through H). In some, but not all, of these asters the centrioles were orthogonal to one another as found in vivo. Many more MTs were associated with asters obtained from the longer incubation (15 min) than the shorter one (4 min) (Figs. 1 and 3).

We conclude that between 4 and 15 min of incubation, centriole duplication oc-

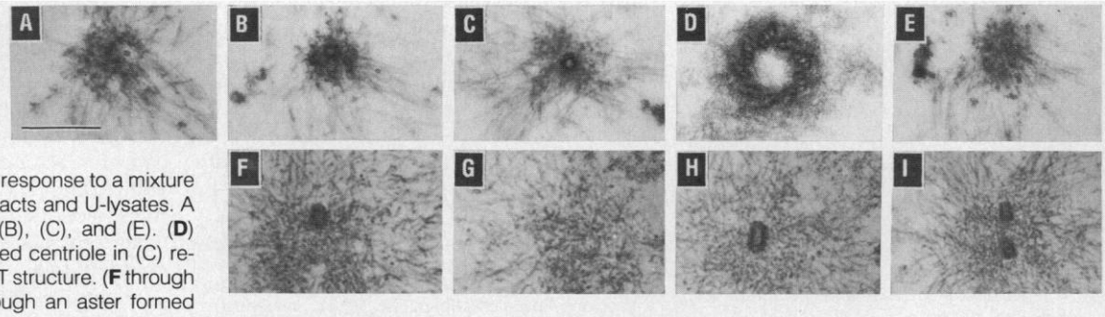

Fig. 3. High-voltage electron micrographs of serial 0.25-μm-thick sections through asters formed in *Spisula* oocyte extracts (*21*). (**A** through **C** and **E**) Four serial sections through an aster formed after 4 min in response to a mixture of equal volumes of HSA-extracts and U-lysates. A single centriole is visible in (B), (C), and (E). (**D**) High magnification of sectioned centriole in (C) reveals the typical nine-triplet MT structure. (**F** through **H**) Three serial sections through an aster formed after 15 min. Two centrioles are visible in (F) and (H) which are separated in (G) by one serial section. (**I**) Single section through a second aster incubated for 15 min reveals two centrioles present in the same section. More MTs were associated with the asters incubated for 15 min than with those incubated for 4 min. Bar, 1.0 μm; all panels except (D), same magnification.

curred in these lysate mixtures. Further, the first centriole to appear during astral formation in our cell-free system must have assembled de novo within 4 min after HSA-extracts and U-lysates were mixed. This conclusion is based on the fact that neither centrosomes (*13–15*) nor centrioles (*16, 17*) have been found in unactivated *Spisula* oocytes or in U-lysates (*18*). In addition, asters in lysates of *Spisula* oocytes prepared 2.5 min after activation did not contain centrioles, whereas those prepared 4.5 min after activation contained a single centriole (*18*). Finally, we did not find asters or centrioles in either HSA-extracts or U-lysates, even when we incubated them for 15 min at room temperature.

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20. Oocytes were obtained and parthenogenetically activated with KCl as previously described (*13, 14, 19*). Four minutes after activation, lysates were prepared and asters isolated (*19, 21*). For analysis of spindle-associated asters, oocytes were activated and incubated at 23°C with gentle stirring. Fifteen minutes after activation oocytes were centrifuged and washed with glycerol buffers (*19*), and spindles were isolated by resuspension in MT stabilizing buffer (MSB) [20 mM Mes, 10 mM EGTA, 5 mM $MgSO_4$, and 20% glycerol (pH 6.3 to 6.5)] containing 1% Triton X-100 or NP-40. Samples were centrifuged at 1000*g* for 10 min. The pellets were resuspended in MSB, centrifuged again, fixed for 15 to 30 min in MSB containing 1% glutaraldehyde, washed in distilled H_2O, postfixed in 1% OsO_4, and embedded in Epon. Serial 0.25-μm sections were prepared with a diamond knife, stained with uranyl acetate followed by lead citrate, and examined with the Wadsworth Center's AEI-EM7 high-voltage electron microscope operated at 800 kV (*7, 22*).
Lysates were prepared, and aster content was analyzed as described (*19*). To remove asters and centrosomes, we centrifuged lysates prepared 4 min after activation (A/4-lysates) at 29,000*g* at 4°C for 15 min. The clarified middle cytosolic layer (HSA-extract) was collected and analyzed for aster content with hexylene glycol (*19*). This procedure was repeated until the HSA-extract was unable to form asters. Aliquots of lysates and HSA-extracts were removed, and proteins analyzed by one-dimensional gel electrophoresis (*23*). Proteins were separated on 7.5% acrylamide gels, and lanes were loaded on the basis of equal volume. We prepared U-lysates by washing oocytes in 1 M glycerol solution (*14, 19*), suspending them in 1 M glycerol containing 10 mM NaH_2PO_4 (pH 8.0) for 1 min, and washing them in aster buffer [20 mM Pipes, 100 mM NaCl, and 5 mM $MgSO_4$ (pH 7.2)]. After centrifugation and aspiration, we lysed oocytes by gentle vortexing and kept them on ice until use. For IMF, 10-μl aliquots of the experimental mixture were diluted into 100 μl of MSB-containing detergent and fixed by the addition of 3 ml of phosphate-buffered saline (pH 7.2) containing 0.5% glutaraldehyde and 0.4% Triton X-100, centrifuged onto polylysine-coated cover slips, and processed as described (*24*) with monoclonal antibody to tubulin (TU27) and rhodamine secondary antibodies (Calbiochem). Samples were photographed with an Axiophot microscope (Zeiss). For HVEM, aliquots of mixtures of HSA-extract and U-lysate were diluted 1:10 to 1:20 in MSB-containing detergent and centrifuged at 1000*g* for 10 min. The pellets were fixed, embedded, sectioned, and analyzed as described (*20, 22*).
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Constructing Proteins by Dovetailing Unprotected Synthetic Peptides: Backbone-Engineered HIV Protease

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Backbone-engineered HIV-1 protease was prepared by a total chemical synthesis approach that combines the act of joining two peptides with the generation of an analog structure. Unprotected synthetic peptide segments corresponding to the two halves of the HIV-1 protease monomer polypeptide chain were joined cleanly and in high yield through unique mutually reactive functional groups, one on each segment. Ligation was performed in 6 molar guanidine hydrochloride, thus circumventing limited solubility of protected peptide segments, the principal problem of the classical approach to the chemical synthesis of proteins. The resulting fully active HIV-1 protease analog contained a thioester replacement for the natural peptide bond between Gly^{51}-Gly^{52} in each of the two active site flaps, a region known to be highly sensitive to mutational changes of amino acid side chains.

Protein engineering has been mainly carried out by site-directed mutagenesis (*1*) or other techniques of genetic manipulation (*2*) and, with limited exceptions (*3*), has been restricted to substitutions based on the genetically coded amino acids. The chemical synthesis approach (*4*) to the systematic variation of protein structure is in principle a much more general one and offers great flexibility in the incorporation of non-coded moieties ranging from unnatural amino acids (*5*) to fixed elements of three-dimensional (3-D) structure (*6*).

An ideal approach to protein engineering would involve the chemical ligation of readily available, large unprotected synthetic peptide segments to give the modified polypeptide chain corresponding to the target protein or functional domain. Here we describe a method for the preparation of protein analogs in which the act of joining two peptides generates an analog structure within the protein molecule. Such an approach allows the use of highly selective chemistries for the ligation reaction in a form of chemical dovetailing in which the reacting moieties on the segments to be joined have reactivities tailored to one another. This approach does away with the need for protecting groups for other functionalities present in the protein molecule (*7, 8*) and provides a flexible, general route to the total chemical synthesis of a wide range of protein analogs, including a new class of protein analog, namely, "backbone-engineered" proteins, in which the peptide bond has been replaced for structure-activity studies. Application of this approach is exemplified by the preparation of a fully functional backbone-engineered enzyme.

Human immunodeficiency virus–1 protease (HIV-1 PR) is a virally encoded en-

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