which uses an excess of mercury, oxidation might occur through impurities, but also by the reduction of mercury.

Synthesis of compound **1** shows the possibility to use Zintl ions as building blocks to construct complex structures. In agreement with the structural results and charge allocation, crystals of **1** shows no signal in the EPR spectrum.[18] Partial oxidation or reduction of the polymer chain—which corresponds to an insertion of paramagnetic clusters into the chain—should lead to interesting magnetic properties. Each homoatomic cluster of the one-dimensional polymer in **1** has two more reactive sites. In principle, these sites hold the possibility to build two-dimensional polymers by repeated oxidative coupling in presence of atoms able to build bridges. In attempts to prepare analogues of **1**, syntheses using Sn_9 clusters instead of Ge_9, up to now, simply lead to the decomposition of the solution and formation of elementary tin. A comparable synthesis to link Sn_9 clusters with Te atoms leads to the break down of the cluster and formation of the heteroatomic ions $[Te_2Sn(\mu_2\text{-}Te)_2SnTe_2]^{4-}$ and $[Sn(\mu_2\text{-}Te)_3Sn]^{2-}$.[19]

Experimental Section

The synthesis of compound **1** was carried out under argon atmosphere using a glove-box or Schlenk line. Toluene (Merck) and en (Merck) were dried over CaH_2 (Fluka), freshly distilled and degassed. The binary phase of the formal composition "K_4Ge_9" was prepared by high-temperature reaction (650 °C) from the elements K and Ge (Chempur) in the ratio 4:9. K_4Ge_9 (200 mg, 0.25 mmol) was dissolved in en (3 mL) in a Schlenk tube and stirred for 5 min in an ultrasonic bath. The resulting dark green solution was filtered onto to Hg (300 mg, 1.5 mmol). Afterwards the reaction mixture was treated for 20 min in an ultrasonic bath, filtered, and added to [2.2.2]crypt (376 mg, 1 mmol). The resulting solution was layered with toluene (3 mL). After about three days compound **1** crystallized as red brown columnar crystals with a yield of 90 mg (20 % with respect to K_4Ge_9). Elemental analysis (%) calcd: C 26.61, H4.91, N 6.21; Found: C 26.82, H 4.95, N 6.04.

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[9] Crystal structure determination: Crystals of **1** were mounted in glass capillaries. Structure solution (SHELXS-97) and refinement (SHELXL-97) was carried out with direct methods and full-matrix least-square methods based on F^2 for all non-hydrogen atoms except those of the en molecules, with anisotropic displacement parameters. Crystal dimensions $0.17 \times 0.42 \times 0.46$ mm^3, lattice constants at 150 K: $a = 24.994(5)$, $b = 16.242(3)$, $c = 16.062(3)$ Å, $\beta = 103.51(3)°$, $V = 6340(2)$ Å^3; space group $P2_1/c$ (No. 14), $Z = 4$, $\rho_{calcd} = 1.891$ g cm^{-3}, $\mu = 6.800(2)$ mm^{-1}; data collection: STOE-IPDS2, $Mo_{K\alpha}$-radiation, $2\Theta_{max} = 50.02°$ (imaging-plate distance 120 mm); of 49959 total reflections, 11123 were independent ($R_{int} = 0.108$); $R_1 = 0.061$ and $Rw = 0.102$ for 609 parameters and 8619 reflections with $I > 2\sigma(I)$, $R_1 = 0.081$ and $Rw = 0.108$ for all data, respectively. CCDC-182906 (**1**) contains the supplementary crystallographic data for this paper. These data can be obtained free of charge via www.ccdc.cam.ac.uk/conts/retrieving.html (or from the Cambridge Crystallographic Data Centre, 12, Union Road, Cambridge CB2 1EZ, UK; fax: (+44) 1223-336-033; or deposit@ccdc.cam.ac.uk).

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A Further Step towards Single-Molecule Sequencing: *Escherichia coli Exonuclease* III Degrades DNA that is Fluorescently Labeled at Each Base Pair**

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The international race to sequence the human genome as well as the genomes of other model organisms encouraged efforts to realize "single-molecule sequencing"—an idea which nourishes the hope to simplify and speed up the task of sequencing DNA segments as long as 50000 base pairs (bp) and joining the sequence information of genome fragments.[1–5] The different strategies to realize single-molecule sequencing are based on that single fluorescent molecules can be identified within milliseconds,[6] and they combine sequential enzymatic hydrolysis of individual DNA molecules with subsequent identification of the released monomers by their

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fluorescence characteristics, either wavelengths, or fluorescence lifetimes, or both.[7,5]

Two unusual enzymatic activities are required for this technique, 1) the complete and faithful synthesis of DNA copies exclusively from fluorescently labeled analogues of the four types of bases (A, G, C, and T), and 2) the exonucleolytic degradation of the completely labeled DNA. Recently, we presented a solution to the problem of complete enzymatic labeling by identifying the wildtype Klenow fragment (KF) of *E. coli* DNA polymerase I as the first natural polymerase which retains full activity and fidelity in the sole presence of rhodamine-labeled deoxynucleoside triphosphates.[8] We demonstrated that the complete substitution of all the pyrimidine bases of one DNA strand and thus, complete labeling of every base pair by the respective fluorescent analogues can be achieved using KF in a primer-extension reaction.[9]

That a natural DNA polymerase exhibited full performance with bulky rhodamine-labeled substrates and was also capable of accommodating the sterically and electronically demanding DNA product stimulated us to screen a series of natural exonucleases for activity on substrate DNA that was completely rhodamine-labeled at all the pyrimidine bases of one strand (fluorescent nucleotides: Tetramethylrhodamine-dUTP, and Rhodamine-Green-dCTP). In preliminary experiments which were based on the online monitoring of fluorescence intensity in a fluorimeter (LS-50; Perkin-Elmer) we assessed the acceptance of completely rhodamine-labeled DNA by bacteriophage λ exonuclease, snake venom phosphodiesterase, as well as two DNA polymerases known to possess $5'\rightarrow3'$ exonuclease activity, bacteriophage T7 DNA polymerase, and KF. Since DNA that harbors a very high density of rhodamine labels usually is nonfluorescent (except those sequences which contain homopolymeric regions of A:T (A:U) base pairs), we expected to observe a substantial increase in fluorescence intensity upon release of fluorescent monomers by an exonucleolytically active enzyme. However, the fluorescence behavior of the reaction systems studied did not change, which indicated that none of the enzymes showed activity with the highly modified substrate, no hydrolytic reaction occurred. Because labeled DNA tends to aggregate in aqueous solution, we added various organic solvents (e.g. acetonitrile, or DMSO) to a maximum of 10% (v/v). Although these were known to increase the solubility of the highly modified DNA[9] they did not enhance the targeted reaction because their denaturing, or toxic effect on the enzymes prevailed. An important hint towards a solution of this problem came from the observation that a high degree of fluorescence labeling could promote the transition of a portion of right-handed B-form DNA to left-handed Z-form DNA:[9] In accord with observations made by Ogilvie and Hruska for snake venom phosphodiesterase,[10] we assumed that the labeled DNA which possibly contains syn–oriented nucleosides was not accepted as a substrate by the enzymes studied. In further tests, we employed *E. coli* exonuclease III because this enzyme had been useful in searching for potential Z-DNA regions within genomic DNA.[11] Indeed, we observed a substantial increase in rhodamine fluorescence upon addition of exonuclease III to an aqueous solution of labeled DNA (no co-solvents present). In attempting to optimize the performance of this reaction, we tested a variety of additives, such as bovine serum albumin (BSA), glycerol, spermidine, sarcosine, DMSO, or Triton-X-100, all of which did not increase the turnover of substrate DNA. The addition of a maximum of 25% 1,4-dioxane, however, enhanced the DNA-degradation reaction substantially by supporting the solution of the dye-labeled DNA without affecting the natural enzyme.

Since sequential degradation of completely rhodamine-labeled DNA by exonuclease III could be the key to a future single-molecule sequencing, we examined the action of this enzyme in detail. Using equilibrium kinetic analysis, we varied a series of rate-determining factors, which include enzyme concentration, temperature, or presence of organic co-solvent, and we compared the results gained with the labeled DNA to a standard reaction of exonuclease III and natural DNA of the same sequence (with only rhodamine label at the $5'$-terminal for detection). Quantitative fragment analysis by using an automated fluorescence-based sequencing system (ABI 373A) enabled us to determine fragment lengths of educt and product(s) relative to an internal standard, as well as fluorescence intensities (relative concentrations) of all the fragments present. Evaluation of the electropherograms permitted the estimation of apparent hydrolysis rates in terms of nucleotides per second, as well as the determination of second-order reaction rates (see Supporting Information for details).

Processivity: The most striking difference between the degradation reactions of natural and completely dye-labeled substrate with or without dioxane could be recognized by visual inspection of the electropherograms. We observed that exonuclease III degraded the fluorescent substrate DNA in dioxane-containing solutions without showing intermediates (that is, processively) at the time intervals chosen (earliest point = 0.3 min after addition of enzyme). Even at a 280-fold molar excess of exonuclease III over substrate, this degree of processivity was not achieved with natural DNA or in the absence of 1,4-dioxane. Instead, we observed—in accordance with other investigations[12]—that progressively shortened intermediates appeared with increasing reaction time (usually more than one species centering around a predominant fragment length) while the respective precursor(s) disappeared (*distributive* mode).

High processivity of exonuclease III at rhodamine-labeled DNA is related to the reaction conditions being different from the "natural reaction". The limited availability of substrate within the aqueous phase requires the presence of solubility-mediating 1,4-dioxane. Association of enzyme and substrate to the active complex therefore might take place in "solvent cages" where the reaction into products comes to an end before enzyme and substrate dissociate.

Apparent rate of hydrolysis: The activity of exonuclease III at fluorescently labeled DNA appeared to be independent of enzyme concentration and temperature when the reaction was performed with the organic co-solvent. Because no intermediates were present, and because the electropherograms of samples taken after increasing periods of time differed in the relation of educt and product only, a maximal rate was presumed. According to this assumption, the rate of

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Table 1. Apparent rate of hydrolysis, $\bar{\nu}$, and processivity of exonuclease III as derived by visual evaluation of electropherograms.[a]

Entry	DNA label	$[E_0]$ [U]	T [°C]	1,4-dioxane	Substrate turnover [%]	t [min]	$\bar{\nu}$ [nt s^{-1}]	Processivity
1	TMR, RG	5	37	+	26	0.3	16.5	+
2	TMR, RG	50	37	+	72	0.3	16.5	+
3	TMR, RG	250	37	+	67	0.3	16.5	+
4	TMR, RG	50	10	+	38	0.3	16.5	+
5	TMR, RG	50	20	+	68	0.3	16.5	+
6	TMR, RG	50	37	+	72	0.3	16.5	+
7	TMR, RG	25	20	+	56	0.3	16.5	+
8	TMR, RG	25	20	−	15	5.0	1.1	−
9	none[b]	25	20	−	98	0.2	4.1	−

[a] TMR = tetramethylrhodamine; RG = rhodamine–green. [b] 5′-Terminal RG label.

processive hydrolysis reached a value of 16.5 nucleotides per second (nt s^{-1}). Intermediate degradation products of both, dye-labeled and natural DNA, arose when no 1,4-dioxane was present. Whereas natural DNA was hydrolyzed at a rate of $\approx$ 4 nt s^{-1} (this value is in good agreement with published data of $\approx$5 nt s^{-1} at 37 °C),[12,13] the rate decreased to about 1 nt s^{-1} with dye-labeled DNA.

Enzyme concentration and temperature, however, influenced the extent of substrate turnover at the time of first product appearance. This finding reflects that 1) an increase of temperature enhances the solubility of substrate DNA, and 2) elevated enzyme concentrations increase the probability for the association of enzyme and substrate (Table 1). Of note is that the activity of exonuclease III is strongly dependent on the incubation temperature under natural conditions (rate duplication every 6 °C between 22 and 46 °C).[12]

Regarding our aim to investigate the biochemical requirements for a single-molecule sequencing, we now can conclude that the fundament has been built: Although it will probably be necessary to label all four bases of one strand of DNA with a fluorescent label, and to then digest the labeled strand, it could, in principle, be sufficient to only label two nucleotides, and then repeat the experiment with all possible permutations.[7,14] This "minimal", or two-color, approach could be accomplished with our findings that DNA can be copied into two daughter strands with complete tagging of each pyrimidine base, and that the heteroduplex DNA with one strand of natural origin, the complementary strand being labeled, serves as a template for the sequential hydrolytic degradation by exonuclease III of *E. coli* if 1,4-dioxane is added. The solvent not only facilitates the solubility of the labeled DNA but also changes the microscopic behavior of the enzyme from the distributive to a highly processive mode. Thus, we are confident that the "proof-of-concept" of single-molecule sequencing will now become feasible.

Experimental Section

Steady-state kinetic analysis: A solution of dsDNA template (40 fmole) with one strand completely rhodamine-labeled at each pyrimidine (ca. 370 bp; fragment of HIV reverse transcriptase coding sequence) was prepared in reaction buffer (75 μL) composed of Tris-HCl (66 mM, pH 8.0), $MgCl_2$ (0.66 mM), dithiothreitol (DTT; 50 mM), BSA (0.5 mg mL^{-1}), and 25% (v/v) 1,4–dioxane. Exonucleolytic degradation of the substrate was started by addition of exonuclease III (2 μL; dilutions containing the amounts of enzyme units as indicated in Table 1; United States Biochemicals) and incubation at 20 °C. Samples (7 μL) were taken prior to the addition of enzyme (0 min), as well as after 0.2, 2, 5, 10, 15, 20, 30, 60, 120 min, and the reaction was stopped with ethylendiaminetetraacetate (EDTA; 1 μL; 0.5 M). Following lyophilization, samples were dissolved in loading buffer (4 μL; formamide:25 mM EDTA = 5:1 (v/v) containing 50 mg mL^{-1} blue dextrane), supplied with 1 μL internal fragment length standard ROX–1000 (Applied Biosystems), and analyzed using sequencing gels (6% polyacrylamide, 8.3 M urea) with resolution on an ABI 373A sequencer. Fragment lengths and their relative concentrations were determined by employing GeneScan 672 software (ABI).

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