emphasize the important role of the His 244 moiety in reducing the energy barrier to radical rearrangement.

The unraveling of the mechanism of action of methylmalonyl-CoA mutase demonstrates an effective synergistic interplay between theory and experiment. The present study complements previous experimental[4, 6, 7, 9] and theoretical[3] investigations in this respect. It provides a qualitative explanation for the magnitude of the measured rate reduction associated with removal of an important active-site residue (His 244), which may be ascribed to a change in the rate-determining step. Our results also re-emphasize the importance of His 244 in catalysing the radical rearrangement step.

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[1] For recent reviews, see, for example: a) B. T. Golding, W. Buckel in *Comprehensive Biological Catalysis*, *Vol. 3* (Ed.: M. L. Sinnott), Academic Press, London, **1997**, pp. 239 – 259; b) P. R. Evans, F. Mancia in *Vitamin B_{12} and B_{12}-Proteins* (Ed.: B. Kräutler, D. Arigoni, B. T. Golding), Wiley-VCH, Weinheim, **1998**, pp. 217 – 226; c) R. Banerjee, S. Chowdhury in *Chemistry and Biochemistry of B_{12}* (Ed.: R. Banerjee), Wiley, New York, **1999**, pp. 707 – 730.

[2] a) J. Halpern, *Science* **1985**, *227*, 869 – 875; b) B. Beatrix, O. Zelder, F. K. Kroll, G. Orlygsson, B. T. Golding, W. Buckel, *Angew. Chem.* **1995**, *107*, 2573 – 2576; *Angew. Chem. Int. Ed. Engl.* **1995**, *34*, 2398 – 2401.

[3] a) D. M. Smith, B. T. Golding, L. Radom, *J. Am. Chem. Soc.* **1999**, *121*, 1383 – 1384; b) D. M. Smith, B. T. Golding, L. Radom, *J. Am. Chem. Soc.* **1999**, *121*, 9388 – 9399.

[4] a) F. Mancia, N. H. Keep, A. Nakagawa, P. F. Leadlay, S. McSweeney, B. Rasmussen, P. Bosecke, O. Diat, P. R. Evans, *Structure* **1996**, *4*, 339 – 350; b) F. Mancia, P. R. Evans, *Structure* **1998**, *6*, 711 – 720; c) N. H. Thomä, T. W. Meier, P. R. Evans, P. F. Leadlay, *Biochemistry* **1998**, *37*, 14368 – 14393; d) F. Mancia, G. A. Smith, P. R. Evans, *Biochemistry* **1999**, *38*, 7999 – 8005.

[5] The Protein Data Bank (PDB) code for the structure shown in Figure 1 is 5req. For additional information, see ref. [4c].

[6] N. Maiti, L. Widjaja, R. Banerjee, *J. Biol. Chem.* **1999**, *274*, 32733 – 32737.

[7] N. Thomä, P. R. Evans, P. F. Leadlay, *Biochemistry* **2000**, *39*, 9213 – 9221.

[8] a) J. Rétey, *Angew. Chem.* **1990**, *102*, 373 – 379; *Angew. Chem. Int. Ed. Engl.* **1990**, *29*, 355 – 361; b) J. Rétey in *Chemistry and Biochemistry of B_{12}* (Ed.: R. Banerjee), Wiley, New York, **1999**, pp. 271 – 287.

[9] T. W. Meier, N. H. Thomä, P. F. Leadlay, *Biochemistry* **1996**, *35*, 11791 – 11796.

[10] G3(MP2)-RAD(p) calculations have been successfully employed to study related B_{12}-mediated rearrangements: a) S. D. Wetmore, D. M. Smith, L. Radom, *J. Am. Chem. Soc.* **2000**, *122*, 10208 – 10209; b) S. D. Wetmore, D. M. Smith, B. T. Golding, L. Radom, *J. Am. Chem. Soc.* **2001**, *123*, 7963 – 7972.

[11] These data represent a slight improvement over results from G2(MP2,SVP)-RAD(p) studies and a significant improvement over energetics reported previously that were calculated at the B3-LYP level.[3] We also note that re-investigation of the potential-energy surfaces for **1**, **2** and **3** revealed additional conformers which more closely resemble the substrate conformation in the crystal structure. The new structure for **1** is also lower in energy than that previously reported.[3] For consistency, we report all energy barriers with respect to these new conformers. For full details of the geometries, see the Supporting Information.

[12] Support for the appropriateness of our model is also provided by the good agreement between the calculated separation of the carbonyl oxygen atom of the 3-propanal radical and the nitrogen atom in the (protonated) imidazole ring (2.68 Å) or NH_4^+ group (2.59 Å) and the corresponding O ··· N distance in the crystal structure (2.7 Å, Figure 1). The O ··· N distance in the more elaborate NH_4^+-bonded model **3** is 2.59 Å.

[13] We note good agreement between energy barriers for hydrogen abstraction from methane by a methyl radical calculated at 298 K and including a tunneling correction at G3(MP2)-RAD(p) (52.7 kJ mol^{-1}) and W1 (49.9 kJ mol^{-1}) levels of theory (see refs. [14] and [15]). W1 is a high-level theoretical procedure that has been designed with the goal of reproducing experimental results to within 1 kJ mol^{-1} (see: J. M. L. Martin, G. de Oliveira, *J. Chem. Phys.* **1999**, *111*, 1843 – 1856).

[14] The energy barriers to hydrogen abstraction are reported with respect to the infinitely separated reactants.

[15] We have approximated the contribution of tunneling to the energy barriers to hydrogen transfer using Bell's tunneling correction (see: R. P. Bell, *The Tunnel Effect in Chemistry*, Chapman and Hall, London, **1980**, pp. 60 – 63). We use B3-LYP/6-31G(d,p) frequencies and G3(MP2)-RAD(p) energy barrier heights to calculate the tunnelling factor. The appropriateness of this analysis is currently being investigated in more detail.

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Kinetic Monitoring of Self-Replicating Systems through Measurement of Fluorescence Resonance Energy Transfer

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KEYWORDS:

autocatalysis · self-replication · fluorescence spectroscopy · kinetics · oligonucleotides

Chemical self-replicating systems have been the subject of various investigations over the last 15 years.[1] In the simplest implementation, two starting materials A and B react with each other to give a self-complementary template molecule C through an autocatalytic reaction cycle.[2] The latter cycle involves the termolecular complex ABC between the template and its precursors as well as the template duplex C_2 and can be described by the gross reaction equation $A + B + C \rightleftharpoons ABC \rightarrow C_2 \rightleftharpoons 2C$. Three basic classes of template molecules have been utilized so far, namely oligonucleotide analogues,[3] peptides,[4] and artificial templates.[5] Despite the structural diversity of these templates, the kinetics of their autocatalytic synthesis were always found to be limited by product inhibition caused by the dimerization of template C.[6] Consequently, these systems typically exhibited the so-called square-root law and autocata-

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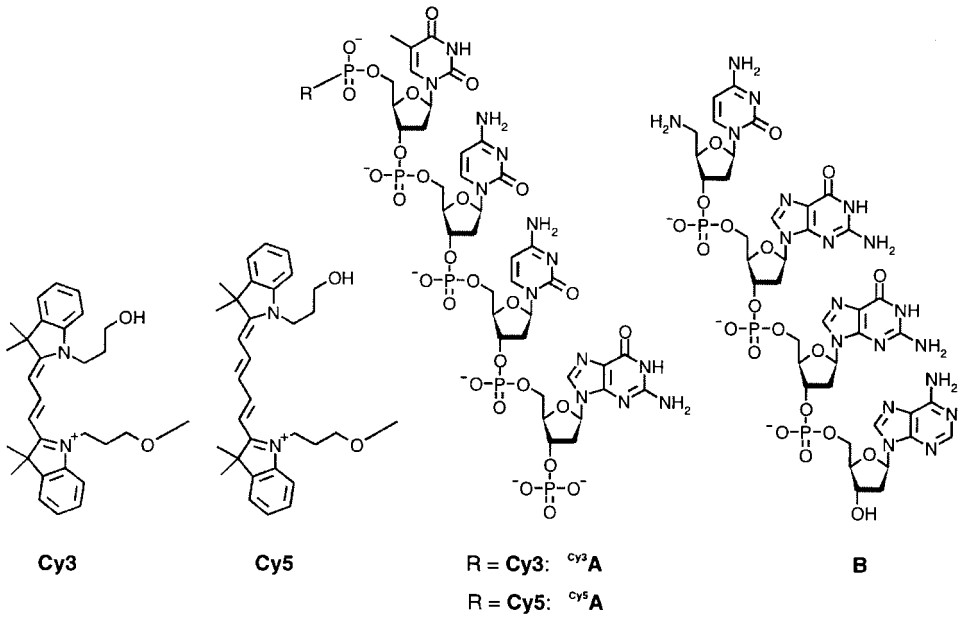
Supporting information for this article is available on the WWW under http://www.chembiochem.com or from the author.

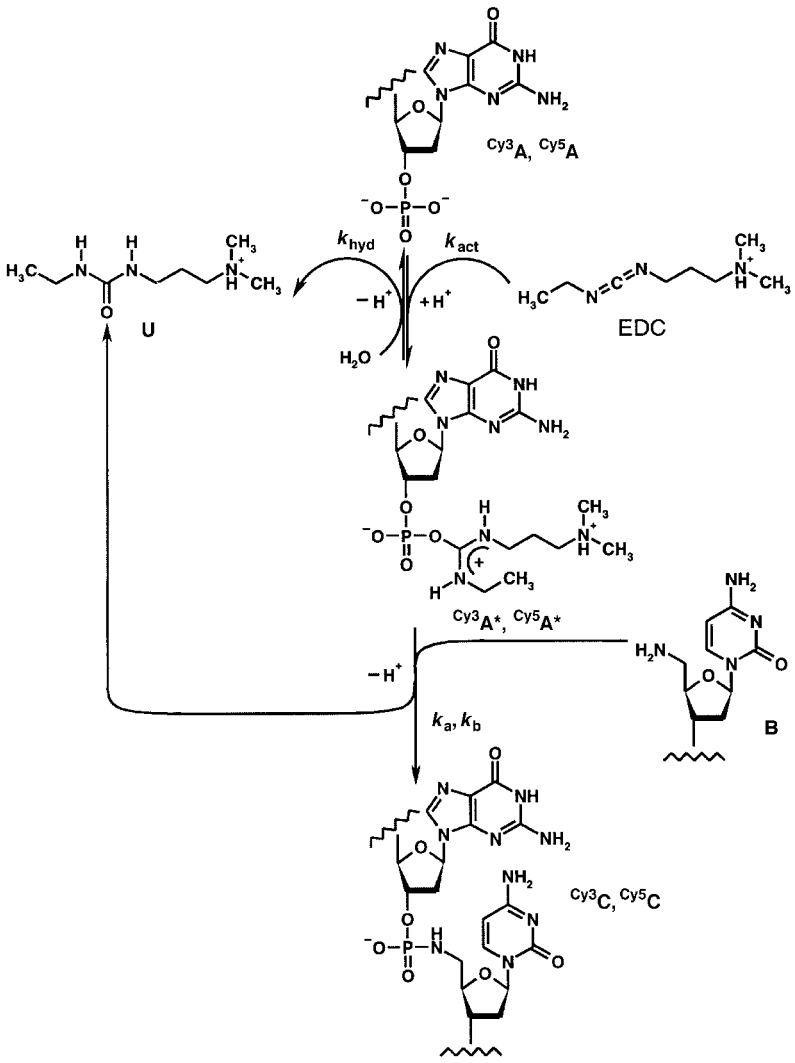
lytic growth that was not exponential, but parabolic. Parabolic growth dynamics also underlie more complex systems where product inhibition is an issue, such as cross-catalytic systems or reaction networks with a multitude of autocatalytic or cross-catalytic feedback loops.[7] However, exponential growth has been described as the prerequisite for possible experimental approaches aiming to realize molecular evolution based on a Darwinian "survival of the fittest" model.[8] Recently, exponential growth was realized in the laboratory by means of a stepwise replication procedure (called SPREAD[9]) that involves the covalent attachment of templates to surfaces. Most recently, theoretical evidence was found that the nonstepwise (autonomous) coupling of chromatography and autocatalytic synthesis may lead to Darwinian selection and, more generally speaking, to the evolution of evolvability of a chemical system, even in the presence of product inhibition.[10] Alternative strategies to establish the evolvability may be based on traveling waves,[11] structurally unstable templates,[12] or minimal replicases.[13] Whichever strategy to implement this challenging goal wins in the future, it can be foreseen that rapid techniques for monitoring the chemical kinetics in a parallel fashion are a major prerequisite. As such, high-pressure liquid chromatography (HPLC) and NMR spectroscopy kinetics, by which most of the systems so far described have been studied, are in a sense too laborious. Among several conceivable techniques, those based on fluorescence, fluorescence quenching, and fluorescence resonance energy transfer (FRET)[14] are superior due to their high sensitivity. For example, fluorescence quenching underlies the technique of molecular beacons that has significantly simplified the quantification of product amplification during the polymerase chain reaction.[15] The utilization of fluorescence quenching and FRET has also been reported in kinetic studies[16] of other bioorganic model systems, among which ribozyme reactions[17] and combinatorial approaches[18] are included.



Scheme 1. *Fluorescence dyes Cy3 and Cy5 and tetrameric building blocks for self-replication with measurement of FRET.*

We here report on the online monitoring of chemical replication of self-complementary oligonucleotides by means of FRET. Schemes 1 and 2 show the building blocks and the basic reactions in our study. The two fluorescence dyes, donor Cy3 and acceptor Cy5, were introduced as 5′ labels to give the tetramers ^{Cy3}**A** and ^{Cy5}**A** by standard solid-phase synthesis. In the presence of the water-soluble *N*′-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide (EDC), the 3′-phosphate groups of ^{Cy3}**A** and ^{Cy5}**A** are activated as the respective isourea adducts ^{Cy3}**A*** and ^{Cy5}**A*** and then react with the 5′-amino group of tetramer **B** to yield the corresponding octamers ^{Cy3}**C** and ^{Cy5}**C**, which bear a central 3′,5′-phosphoramidate linkage (Scheme 2). Additionally, the octamer ^{Cy5}**C′**, which has a central 3′,5′-phosphodiester linkage was synthesized as an "external" template, to mimic the effect of the internally synthesized template ^{Cy5}**C**.

The rationale behind the synthesis of two differently labeled tetramers with the same sequence is understood in the context of their reactions. If both ^{Cy3}**A** and ^{Cy5}**A** are allowed to react with tetramer **B**, one expects the simultaneous formation of the octamers ^{Cy3}**C** and ^{Cy5}**C**. Both octamers are self-complementary and, thus, should dimerize to yield the homomolecular duplexes, $(^{Cy3}\mathbf{C})_2$ and $(^{Cy5}\mathbf{C})_2$, as well as the heteromolecular duplex ^{Cy3}**C**:^{Cy5}**C** (Figure 1). The latter complex should be a FRET-active species because, for a conformation of the B-DNA type, the distance between donor Cy3 and acceptor Cy5 is expected to be below 5 nm, above which the efficiency of FRET drops to 50% of its theoretical value for the Cy3 – Cy5 couple.[19] In any case, the efficiency of FRET should scale linearly with the concentration of the complex ^{Cy3}**C**:^{Cy5}**C**. Furthermore, the maximal concentration of this complex is achievable, if the complexes $(^{Cy3}\mathbf{C})_2$ and $(^{Cy5}\mathbf{C})_2$ have equal thermodynamic stabilities. If so, the heteromolecular duplex should reach—for statistical reasons—twice the concentrations of the homomolecular ones. This is exactly the reason why we selected the Cy3 – Cy5 couple instead of other dyes (such as fluorescein – rhodamine). Cy3 and Cy5 only differ by a single C–C bond in the chromophore and, thus, are expected to have very similar influences on the complexes formed. Indeed, a plot of the reciprocal UV-melting temperatures T_m against the logarithm of the oligonucleotide concentration (see Supporting Information) confirmed that the thermodynamic data of the homomolecular duplexes are barely distinguishable. In the following, we used an average value of $\Delta G = -46.9\ \mathrm{kJ\,mol^{-1}}$. From these data it follows that, under the conditions of the self-replication experiments, the association constant K_{homo} for the homomolecular duplexes is $3.2 \times 10^8\ \mathrm{L\,mol^{-1}}$ at 15 °C, whereas $K_{hetero} = 2\,K_{homo}$ for symmetry reasons.



Scheme 2. *Basic reactions taking place in the presence of water-soluble carbodiimide EDC. The 3′-phosphate groups of* ^{Cy3}***A*** *and* ^{Cy5}***A*** *add to EDC to yield the isourea derivatives* ^{Cy3}***A**** *and* ^{Cy5}***A****, *respectively. The activated 3′-phosphate groups are hydrolyzed to reproduce the original phosphate groups. Thus, activation and hydrolysis proceed as quasireversible reactions. Alternatively,* ^{Cy3}***A**** *and* ^{Cy5}***A**** *react with the 5′-amino group of* ***B*** *to give the two possible octameric 3′,5′-phosphoramidates* ^{Cy3}***C*** *and* ^{Cy5}***C****.*

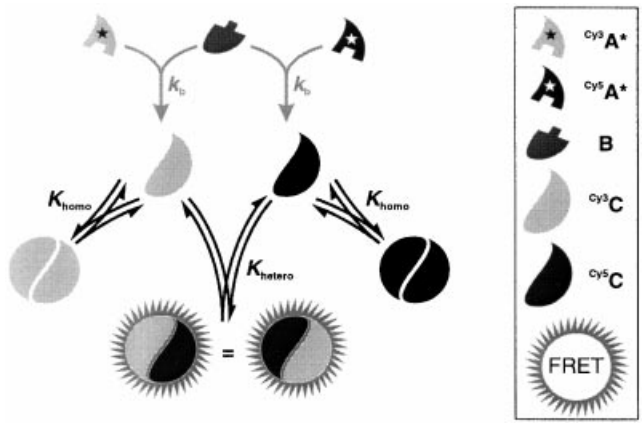


Figure 1. *Nontemplated, simultaneous formation of templates* ^{Cy3}***C*** *and* ^{Cy5}***C*** *from* ^{Cy3}***A****,* ^{Cy5}***A****, and* ***B*** *(k_b) and the equilibrium between heteromolecular (K_{hetero}) and homomolecular (K_{komo}) template duplexes.*

The overall set of reactions expected to occur during and upon simultaneous formation of ^{Cy3}**C** and ^{Cy5}**C** is depicted in Figure 2. There are basically five classes of reactions involved: 1) The reversible formation of the homo- and heteroduplexes, for which thermodynamic data have been derived as outlined above (K_{homo} and K_{hetero}). 2) The reversible formation of termolecular complexes, for which precise thermodynamic data are difficult to obtain[20] (K_{termol}). 3) The irreversible template-directed ligations within termolecular complexes, whose rate constants k_a can only be determined if the concentration of termolecular complexes are known. 4) The nontemplated "background" formation of the octamers, where the determination of rate constant k_b requires knowledge of the degree of activation of 3′-phosphate groups. (k_b applies to the grey reaction pathway.) 5) The activation and hydrolysis of tetramer 3′-phosphate groups, which constitutes a "metabolic" subsystem, whose kinetics could be determined by independent methods. (k_{act} and k_{hyd} are shown in the center of the scheme.) Both reactions contribute to the nonproductive consumption of the carbodiimide EDC, whose spontaneous hydrolysis was reported to be rather slow.[21] Phosphate groups, however, act as a catalyst of carbodiimide hydrolysis, because both the addition of phosphates to the carbodiimide moiety and the hydrolysis of the phosphorylisourea adducts were found to be rather fast processes.[21]

To investigate the nonproductive consumption of carbodimide EDC under conditions close to our FRET experiments, ^{1}H NMR spectroscopy experiments were carried out in which the formation of the respective urea derivative **U** was monitored in the presence of the tetramer 3′-phosphate ^{Bup}TCCG$_P$ (Figure 3; Bup = *n*-butoxyphosphate). The latter compound was synthesized as a model of ^{Cy3}**A** and ^{Cy5}**A**, whose consumption in NMR spectroscopy experiments was thought to be too expensive. ^{Bup}TCCG$_P$ differs from the dye-labeled compounds only with respect to its 5′ end and, thus, should be a good kinetic substitute for reactions taking place at the 3′-phosphate group. The initial concentration of EDC was varied. The experimental production curves for the urea derivative **U** were approximated by nonlinear regression with our SimFit program.[22] The iteration was based on the following reaction model which consists of an activation step (k_{act}), a pseudo first order hydrolysis step (k_{hyd}), and a bimolecular formation of a 3′,3′-pyrophosphate (k_{pp}) as depicted in Equations (1) – (3) (see Supporting Information).

$$^{Cy3/5}\mathbf{A} + \mathrm{EDC} \longrightarrow {}^{Cy3/5}\mathbf{A}^* \qquad (k_{act}) \qquad (1)$$

$$^{Cy3/5}\mathbf{A}^* \longrightarrow {}^{Cy3/5}\mathbf{A} + \mathbf{U} \qquad (k_{hyd}) \qquad (2)$$

$$^{Cy3/5}\mathbf{A}^* + {}^{Cy3/5}\mathbf{A} \longrightarrow {}^{Cy3/5}\mathbf{A}\text{-}\mathbf{A}^{Cy3/5} + \mathbf{U} \qquad (k_{pp}) \qquad (3)$$

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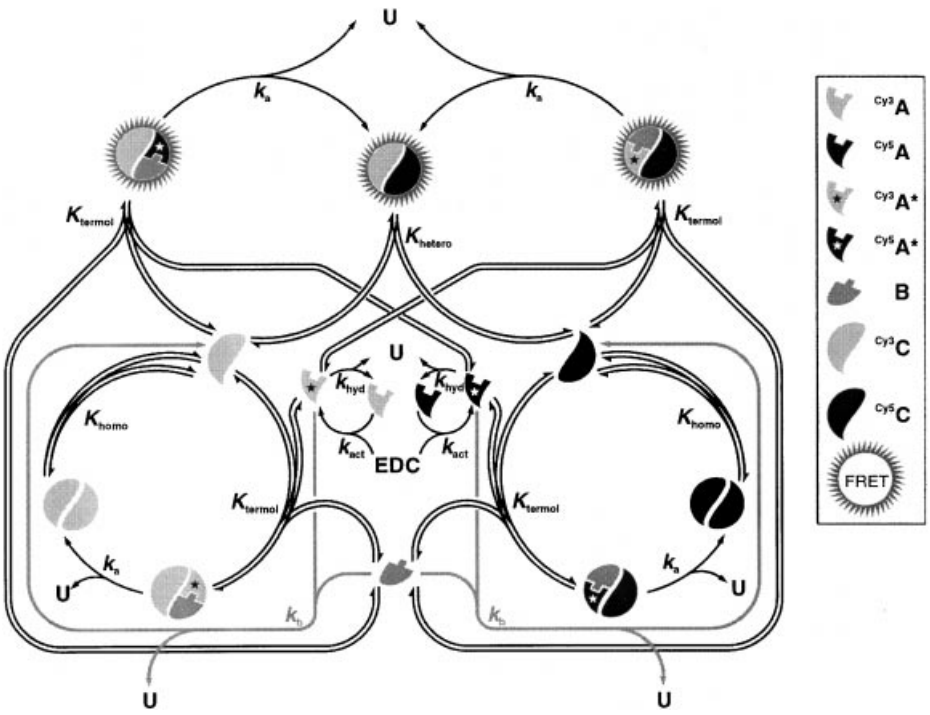

Figure 2. *Overall reaction scheme for the autocatalytic and templated formation of heteroduplexes (upper two cycles) and homoduplexes (lower two cycles) from the amino tetramer and the tetrameric phosphate compounds, whose quasireversible activation is shown by the small inner cycles. Pathways leading to the noncatalyzed formation of template molecules are shown in gray.*

Next, two types of FRET experiment were carried out. In the first type, both ^{Cy3}**A** and ^{Cy5}**A** were allowed to react with tetramer **B** in the absence of template (Figure 2). The formation of the heteroduplex was monitored over 24 hours and resulted directly in a sigmoidal growth curve whose initial development follows a parabola (Figure 4). The concentration of octamers synthesized was determined directly from the decrease of FRET donor absorbance after calibration with a sample in which the octamer concentration arose from 100% conversion of the tetramers. The second type of experiment involved kinetic monitoring of the formation of ^{Cy5}**C** (from just ^{Cy5}**A** and **B**) in the presence of various initial concentrations of "external" template ^{Cy3}**C′**. Figures 5a and b depict the time courses for formation of the heteroduplex and the calculated total concentration of newly formed ^{Cy5}**C**, respectively. The nonlinear curve-fitting of Figure 5 was again performed by means of our SimFit program, and the reaction model shown in Equations (4) – (11) was taken into account.

The rate constants k_{act} and k_{hyd} in Equations (4) and (5) were fixed to the values determined by the ^{1}H NMR spectroscopy kinetic study. Equations (7) and (8) comprise the reversible formation of termolecular complexes and the irreversible chemical ligation events within the latter complexes. As long as reliable thermodynamic data for termolecular complexes

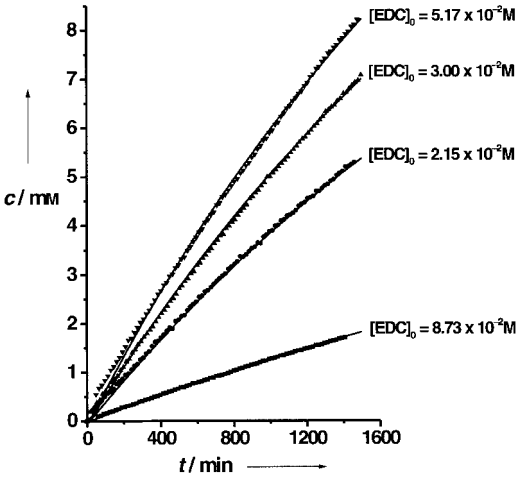

Figure 3. *Time course of the formation of urea* ***U*** *(Scheme 2) during the reaction of tetrameric phosphate ^{Bup}TCCG$_p$ (833 μM solution) in the presence of EDC, the concentrations of which are given in the figure. The experimental concentrations (points) were determined from the well-separated ^{1}H NMR signal of the ethyl CH$_3$ group. The reactions were carried out at 15°C in a buffered solution containing 0.1 M 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) in H$_2$O/D$_2$O/CD$_3$CN (72:8:20) at pH = 7.35. The theoretical time courses (curves) were derived from SimFit calculations based on the reaction model given in the text. The following parameters were determined from these experiments:* $k_{act} = 3.55(\pm 0.02) \times 10^{-3}$, $k_{hyd} = 9.22(\pm 0.30) \times 10^{-4}$, *and* $k_{pp} = 3.03(\pm 0.11) \times 10^{-2}\ M^{-1}\ s^{-1}$.

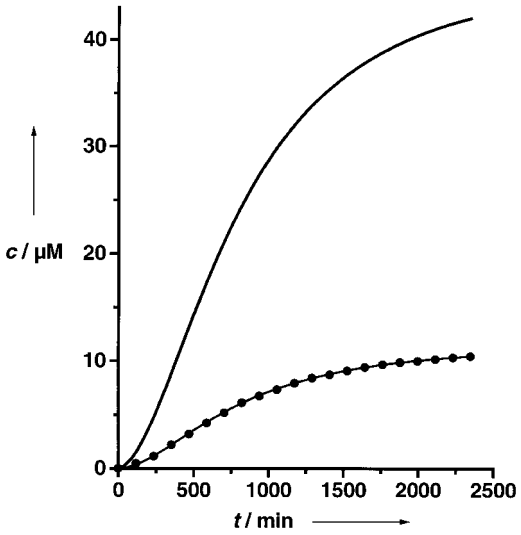

Figure 4. *Time course for the autocatalytic formation of heteroduplexes from a mixture of tetrameric building blocks* ^{Cy3}***A***, ^{Cy5}***A***, *and* ***B***, *(1:1:2, respectively) in the presence of EDC. The points were derived from fluorescence measurements and approximated by a theoretical curve based on the reaction model shown in Figure 2 and the rate parameters estimated by the SimFit program from data shown in Figure 3. The upper curve represents the total concentrations of all octamers formed. Conditions: 40 μM* ^{Cy3}***A***, *40 μM* ^{Cy5}***A***, *80 μM* ***B***, *0.2 M EDC, 0.1 M HEPES; pH 7.35,* T = 15°C.

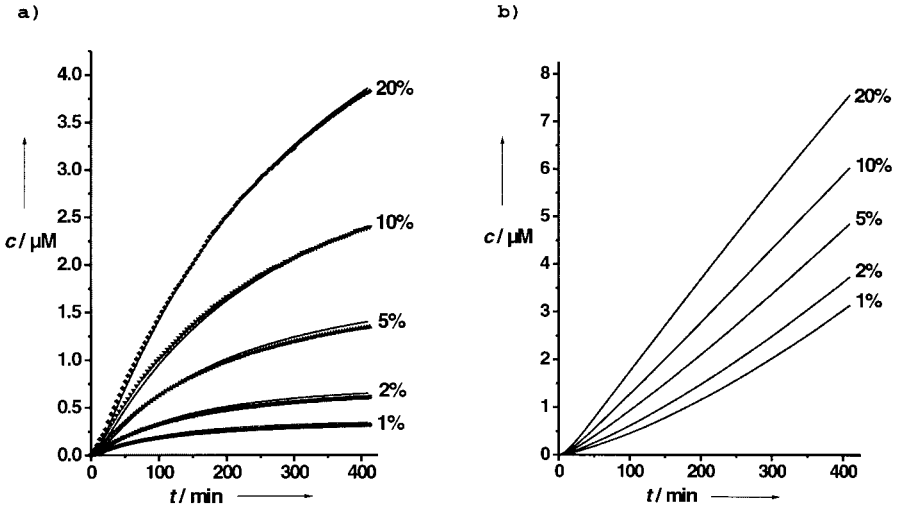

*Figure 5. Time course of (a) heteroduplex formation and (b) total octamer formation in the carbodiimide driven reaction of ^{Cy5}**A** and **B** in the presence of "external" template ^{Cy3}**C'**, at various initial concentrations, which are given in percentages of the concentration of ^{Cy5}**A**. All experimental data (points in (a)) were approximated simultaneously to generate the respective theoretical time courses in (a) and (b). Fitting was based on the respective pathways in Figure 2, with combination of termolecular complex formation and subsequent ligation to pseudo first order reactions as explained in the text. The parameters k_{act} and k_{hyd} were fixed in the calculations to the values given in Figure 3. The association rate constants of duplex formation were set to $10^6\,M^{-1}s^{-1}$ for all cases and the dissociation rate constants were derived from K_{homo} and K_{hetero} as determined from UV-melting studies. Reaction conditions: 40 μM ^{Cy5}**A**, 40 μM **B**; all other conditions as given in the legend of Figure 4.*

^{Cy5}**A** + EDC ⟶ ^{Cy5}**A*** (k_{act} fixed) (4)

^{Cy5}**A*** ⟶ ^{Cy5}**A** (k_{hyd} fixed) (5)

^{Cy5}**A*** + **B** ⟶ ^{Cy5}**C** (k_b variable) (6)

^{Cy5}**A*** + **B** + ^{Cy3}**C'** ⟶ ^{Cy3}**C'**:^{Cy5}**C** (k_a variable) (7)

^{Cy5}**A*** + **B** + ^{Cy5}**C** ⟶ (^{Cy5}**C**)$_2$ (k_a variable) (8)

2 ^{Cy3}**C'** ⇌ (^{Cy3}**C'**)$_2$ (k_{ass}/k_{diss} fixed) (9)

2 ^{Cy5}**C** ⇌ (^{Cy5}**C**)$_2$ (k_{ass}/k_{diss} fixed) (10)

^{Cy3}**C'** + ^{Cy5}**C** ⇌ ^{Cy3}**C'**:^{Cy5}**C** (k_{ass}/ 1/2 k_{diss} fixed) (11)

involving self-complementary template sequences are not available, this assumption of an apparent third-order reaction is the only way to generate a reliable rate constant here. Although the termolecular complexes are expected to be FRET-active species, their concentration is estimated to be significantly lower than that of the template heteroduplex. We were not able to improve our fits significantly, when taking the components of the apparent third-order reaction into account explicitly. A deconvolution of k_a into the ligation rate constant and the corresponding association constant for the termolecular complex is thus the subject of a current investigation with sequences that are not self-complementary. Moreover, interactions of complementary tetramers such as ^{Cy5}**A**:**B** and ^{Cy3}**A**:**B** turned out to be negligible because of their very low melting temperature.

The rate constant k_{diss} in Equations (9) – (11) was taken from the average dimerization constant K_{homo} by assuming a "typical"[23] association rate constant of $10^6\,L\,mol^{-1}\,s^{-1}$. It should be mentioned that we assumed no difference in k_{diss} between external templates having a central 3′,5′-phosphordiester bond and internally synthesized templates bearing a 3′,5′-phosphoramidate bond. This assumption is based on earlier observations with similar hexadeoxynucleotide templates, where the central modification did not influence the overall template effect within the precision of the experiments.[3, 20] Of course, there will be slight differences between Equations (7) and (8) with respect to k_a and also in Equations (9) – (11) with respect to k_{diss}. A deconvolution into individual rate constants does even slightly improve the fits. It is our philosophy, however, not to overstress the kinetic interpretability of our data and to come up with averaged, but meaningful, rate constants.

The two rate constants generated by the FRET study are $k_a = 4.20(\pm 0.02) \times 10^6\,M^{-2}\,s^{-1}$ and $k_b = 6.6(\pm 24) \times 10^{-4}\,M^{-1}\,s^{-1}$, but k_b is not informative. The latter finding is well understood within the context of our theory of minimal replicators.[6] It was shown for the case of parabolic replicators that the noninstructed background synthesis of templates (k_b) may differ by orders of magnitude without giving a noticeable influence on the production curves (see Supporting Information). As long as the nonautocatalytic synthesis of template molecules is only a "background" reaction, one is just able to claim that the autocatalytic synthesis is the dominant one.

We have introduced a FRET-based method for the online monitoring of chemical self-replicating systems. Future work is directed towards the utilization of this technique for the study of cross-catalytic and other systems. As FRET experiments can be carried out on microtiter plates, it is a suitable technique for the rapid screening of factors that exhibit an influence on the dynamics of autocatalytic growth. We are currently undertaking a screening of polyamines and peptides that act as covalent catalysts of the phosphoryl transfer step while selectively stabilizing the termolecular complex. The search for such a "minimal replicase" may lead to a better understanding of how chemical systems were able to manage to create their own evolvability during the origin of life on earth.[25]

Experimental Section

All oligonucleotides were synthesized using standard phosphoramidite chemistry from commercially available phosphoramidites (Pharmacia) on a GeneAssembler Plus (Pharmacia), purified by reversed-phase HPLC (RP-C18, 0 ⟶ 40 % acetonitrile in a 0.1 M aqueous solution

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of ammonium bicarbonate, followed by exhaustive coevaporation with water/ethanol (40:60), and characterized by matrix-assisted laser desorption/ionization mass spectrometry and NMR spectroscopy.[24] Stock solutions of the oligonucleotides were prepared in water/acetonitrile (80:20). The addition of the organic solvent was necessary in order to suppress adsorption of the labeled oligonucleotides onto the surfaces of the Eppendorf tubes used for storage of stock solutions.

For the preparation of the reaction mixtures, aliquots of the respective stock solutions were colyophilized with a SpeedVac concentrator. The pellet was redissolved in 0.1 M 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) buffer (200 μL; Na^+/H^+ form) in water/acetonitrile (80:20) and the solution was transfered to a fluorescence cell (500 μL). The reaction was started by the addition of 1 M EDC (50 μL) to give a carbodiimide concentration of 0.2 M and varying concentrations of oligonucleotides as given in Figures 4 and 5.

Fluorescence data were collected in a stand-alone spectrofluorimeter Kontron SFM 25. The optical path length was set to 1 cm, the excitation wavelength to 500 nm, and the emission wavelength to 557 nm. The overall analysis is based on a linear relationship between the concentration of heteromolecular template duplexes ^{Cy3}**C**:^{Cy5}**C** and the corresponding decrease of donor fluorescence induced through the FRET mechanism. Fluorescence quenching and other side effects, such as bleaching or the hypothetically disturbed fluorescence within homomolecular template duplexes $(^{Cy3}\mathbf{C})_2$ or $(^{Cy5}\mathbf{C})_2$, could be excluded by control experiments under the same reaction conditions and concentrations of building blocks, respectively.

Concentrations of FRET-active species were calculated from the quenching of the fluorescence donor (Cy3) mediated by energy transfer to the acceptor (Cy5) after fluorescence calibration with solutions of heteroduplexes in known concentrations. The latter concentrations were calculated from the given total concentrations of the respective octamers by means of thermodynamic data derived from UV-melting studies (see Supporting Information). Calibration was necessary for a proper determination of the fluorescence offset generated by background fluorescence.

Rate constants were determined from experimental kinetic data with our SimFit program. The command scripts employed are listed in the Supporting Information.

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[1] Reviews: a) B. G. Bag, G. von Kiedrowski, *Pure Appl. Chem.* **1996**, *68*, 2145 – 2152; b) A. Robertson, A. J. Sinclair, D. Philp, *Chem. Soc. Rev.* **2000**, *29*, 141 – 152.

[2] G. von Kiedrowski, *Angew. Chem.* **1986**, *98*, 932 – 934; *Angew. Chem. Int. Ed. Engl.* **1986**, *25*, 932 – 935.

[3] G. von Kiedrowski, B. Wlotzka, J. Helbing, M. Matzen, S. Jordan, *Angew. Chem.* **1991**, *103*, 456 – 459; *Angew. Chem. Int. Ed. Engl.* **1991**, *30*, 423 – 426.

[4] a) D. H. Lee, J. R. Granja, J. A. Martinez, K. Severin, M. R. Ghadiri, *Nature* **1996**, *382*, 525 – 528; b) K. Severin, D. H. Lee, J. A. Martinez, M. R. Ghadiri, *Chem. Eur. J.* **1997**, *3*, 1017 – 1024; c) S. Yao, I. Ghosh, R. Zutshi, J. Chmielewski, *J. Am. Chem. Soc.* **1997**, *119*, 10559 – 10560.

[5] a) T. Tjivikua, P. Ballester, J. Rebek, Jr., *J. Am. Chem. Soc.* **1990**, *112*, 1249 – 1250; b) A. Terfort, G. von Kiedrowski, *Angew. Chem.* **1992**, *104*, 626 – 628; *Angew. Chem. Int. Ed. Engl.* **1992**, *31*, 654 – 656; c) B. Wang, I. O. Sutherland, *Chem. Commun.* **1997**, 1495 – 1496; d) V. C. Allen, D. Philp, N. Spencer, *Org. Lett.* **2001**, *3*, 777 – 780.

[6] G. von Kiedrowski, *Bioorg. Chem. Front.* **1993**, *3*, 113 – 146.

[7] a) D. Sievers, G. von Kiedrowski, *Nature* **1994**, *369*, 221 – 224; b) T. Achilles, G. von Kiedrowski, *Angew. Chem.* **1993**, *105*, 1225 – 1228; *Angew. Chem. Int. Ed.* **1993**, *32*, 1198 – 1201; c) D. H. Lee, K. Severin, Y. Yokobayashi, M. R. Ghadiri, *Nature* **1997**, *390*, 591 – 594; d) D. H. Lee, K. Severin, Y. Yokobayashi, M. R. Ghadiri, *Nature* **1998**, *394*, 101.

[8] E. Szathmáry, I. Gladkih, *J. Theor. Biol.* **1989**, *138*, 55 – 58.

[9] A. Luther, R. Brandsch, G. von Kiedrowski, *Nature* **1998**, *396*, 245 – 248.

[10] G. von Kiedrowski, E. Szathmáry, *Selection* **2000**, *1*, 173 – 179.

[11] J. S. McCaskill, G. J. Bauer, *Proc. Natl. Acad. Sci. USA* **1993**, *90*, 4191 – 4195.

[12] S. Lifson, H. Lifson, *J. Theor. Biol.* **1999**, *199*, 425 – 433.

[13] G. von Kiedrowski in *Gene, Neurone, Qubits & Co.* (Eds.: D. Ganten, E. Meyer-Galow, H. H. Ropers, H. Scheich, H. Schwarz, K. Urban, E. Truscheit), Gesellschaft Deutscher Naturforscher und Ärzte, S. Hirzel Verlag, Stuttgart, **1998**, pp. 123 – 145.

[14] Reviews: a) P. R. Selvin, *Methods Enzymol.* **1995**, *246*, 300 – 334; b) A. Holzwarth, *Methods Enzymol.* **1995**, *246*, 334 – 362; c) R. M. Clegg, *Methods Enzymol.* **1992**, *211*, 353 – 388; d) R. H. Fairclough, C. R. Cantor, *Methods Enzymol.* **1978**, *48*, 347 – 379.

[15] a) S. Tyagi, F. R. Kramer, *Nat. Biotechnol.* **1996**, *14*, 303 – 308; b) S. Tyagi, D. P. Bratu, F. R. Kramer, *Nat. Biotechnol.* **1998**, *16*, 49 – 53.

[16] a) S. S. Ghosh, P. S. Eis, K. Blumeyer, K. Fearon, D. P. Millar, *Nucleic Acids Res.* **1994**, *22*, 3155 – 3159; b) K. P. Bjornson, M. Amaratunga, K. J. M. Moore, T. M. Lohman, *Biochemistry* **1994**, *33*, 14306 – 14316; c) T. A. Perkins, D. E. Wolf, J. Goodchild, *Biochemistry* **1996**, *35*, 16370 – 16377; d) K. K. Singh, R. Parwaresch, G. Krupp, *RNA* **1999**, *5*, 1348 – 1356.

[17] A. Jenne, J. S. Hartig, N. Piganeau, A. Tauer, D. A. Samarsky, M. R. Green, J. Davies, M. Famulok, *Nat. Biotechnol.* **2001**, *19*, 56 – 61.

[18] A. Berkessel, R. Riedl, *Angew. Chem.* **1997**, *109*, 1518 – 1520; *Angew. Chem. Int. Ed. Engl.* **1997**, *36*, 1481 – 1483.

[19] P. I. H. Bastiaens, T. M. Jovin, *Proc. Natl. Acad. Sci. USA* **1996**, *93*, 8407 – 8412.

[20] N. G. Dolinnaya, N. I. Sokolova, O. I. Gryaznova, Z. A. Shabarova, *Nucleic Acids Res.* **1988**, *16*, 3721 – 3738.

[21] a) R. Lohrmann, L. E. Orgel, *Science* **1968**, *161*, 64 – 66; b) N. G. Dolinnaya, A. V. Tsytovich, V. N. Sergeev, T. S. Oretskaya, Z. A. Shabarova, *Nucleic Acids Res.* **1991**, *19*, 3073 – 3080.

[22] D. Sievers, G. von Kiedrowski, *Chem. Eur. J.* **1998**, *4*, 629 – 641.

[23] D. Pörschke, M. Eigen, *J. Mol. Biol.* **1971**, *62*, 361 – 381.

[24] J. Bülle, PhD thesis, Ruhr-Universität Bochum (Germany), **2000**.

[25] During submission of this manuscript, we became aware of the following relevant paper: Y. Xu, N. B. Karalkar, E. T. Kool, *Nature Biotechnol.* **2001**, *19*, 148 – 152.

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