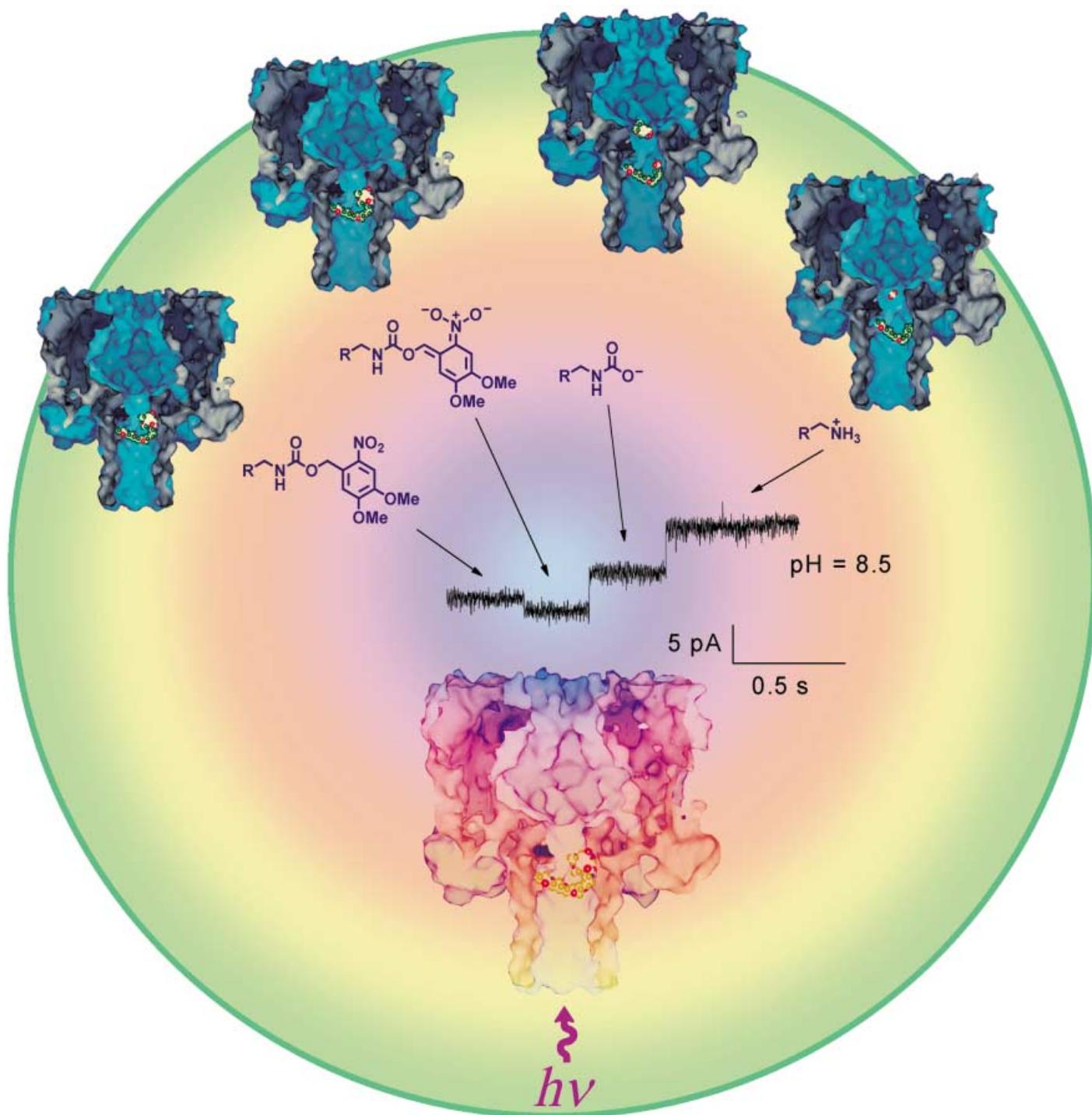


Zuschriften



Eine komplexe strahlungsinitierte dreistufige Einzelmolekülreaktion wurde durch Messung des Ionenstroms durch eine Proteinpore zeitlich verfolgt. Der Strom hängt von der Größe, Form und Polarität der innerhalb der Pore haftenden Reaktanten ab. Mehr zur Messmethode und zur Kinetik dieses Prozesses finden Sie in der Zuschrift von H. Bayley et al. auf den folgenden Seiten.



Kinetics of a Three-Step Reaction Observed at the Single-Molecule Level**

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The ability to observe covalent chemistry at the single-molecule level is expected to yield information about reaction pathways that cannot be obtained from ensemble measurements. Optical, force, and electrical measurements have been used to study the dynamics of individual molecules in solution, and progress has been made in applying these approaches to chemical reactions. For example, single turnovers of enzymes and ribozymes have been examined by optical techniques including fluorescence and light-scattering measurements.^[1-5] Force spectroscopy has been used to investigate the breaking and making of noncovalent bonds, as exemplified by the unfolding and refolding of proteins^[6-10] and RNA.^[3,5,11] Force measurements have also been used to determine the force required to rupture individual covalent bonds at specified loading rates^[12] and to study the photoisomerization of an azobenzene,^[13] although, in the latter work, individual isomerization steps were not resolved.

Electrical measurement of the conductance of nanoreactors comprising single transmembrane pores has several advantages for the observation of covalent chemistry at the single-molecule level; for example, measurements can be carried out with time resolution in the microsecond domain and the procedure does not require a spectroscopic probe such as a fluorophore. Electrical detection at the single-molecule level has been used previously to follow a unidirectional single-step reaction,^[14] to observe a thermal isomerization,^[15,16] and to monitor reversible single-step covalent chemistry.^[17] Finkelstein and co-workers observed the reaction of a cysteine residue in the lumen of the diphtheria toxin pore with $\text{CH}_3\text{SO}_2\text{SCH}_2\text{CH}_2\text{N}(\text{CH}_3)_3^+$. They predicted that the single-molecule approach might be generally useful for the examination of chemical kinetics.^[14] Woolley and co-workers synthesized a gramicidin with a carbamate attached

near the mouth of the pore.^[15,16] They were able to observe the temperature dependence of the rates of *cis-trans* isomerization of the carbamate and thereby determine the activation enthalpies and entropies. In our research group, the reversible reactions of organoarsenic(III) compounds with the thiol group of a single cysteine residue projecting into the lumen of the α -hemolysin (α HL) pore have been examined.^[17] Rate constants for the formation and dissociation of the unstable covalent adducts were obtained.

Here, we have used electrical detection to monitor a multistep reaction at the single-molecule level for the first time. The breakdown of a protected carbamic acid was initiated with light. By this means we determined the rate constants for the cleavage of the 2-nitrobenzyl protecting group after the absorption of a photon and the cleavage of the carbamic acid to give carbon dioxide and a primary amine. Our approach reveals reactive intermediates, uncovers minor pathways, and quantifies the kinetics of steps invisible in conventional kinetic techniques.

An α HL pore was constructed with a 3,4-dimethoxy-6-nitrobenzylcarbamate group covalently attached on the interior wall through an oligo(ethylene glycol) linker (Figure 1a and Supporting Information). In brief, an α HL subunit containing the $\text{Thr}117 \rightarrow \text{Cys}$ mutation was derivatized on the cysteinyl residue with reagent 1. The modified subunit was assembled with wild-type subunits and the heteroheptamer containing a single modified cysteine was separated by electrophoresis. The separation was assisted by the presence of an oligoaspartate tail on the modified subunit. The photochemistry of nitrobenzylcarbamates and related molecules is well developed (Figure 1b). On absorption of a photon the 3,4-dimethoxy-6-nitrobenzyl group rearranges to a nitronate (aci-nitro) intermediate, which has a lifetime of microseconds to seconds depending on the nature of the appended group and the reaction conditions.^[18] The protecting group dissociates as a nitrosoaldehyde to reveal a carbamic acid that loses CO_2 in a pH-dependent step and yields a primary amine. As shown here, the expected chemistry can be observed by planar bilayer recording as three steps in a single-channel current (Figure 2a). The current steps are produced by changes in the size, shape, and charge of the remainder of the molecule tethered within the lumen of the pore.

We examined the pH dependence of both the loss of the nitrobenzyl protecting group and the breakdown of the carbamic acid to demonstrate the potential of the approach (Figure 2b-d). The photoinitiated reaction was carried out around ten times at each of four pH values and the lifetimes of the two intermediates were determined in each case. The reciprocal of the mean lifetime provides an estimator of the rate constant (k) of each step (Figure 2b-d). A statistical analysis yields a confidence interval for the true value of k (see the Supporting Information).

The photolysis of the 2-nitrobenzyl group has been studied extensively because of its utility in organic synthesis, on-chip oligonucleotide synthesis, and the “caging” of small and large molecules for applications in biology. Formation of the nitronate intermediate, by rapid deprotonation of the first-formed nitronic acid, is generally accepted to occur in a few microseconds. We suggest that level 2 (Figure 2a) is the

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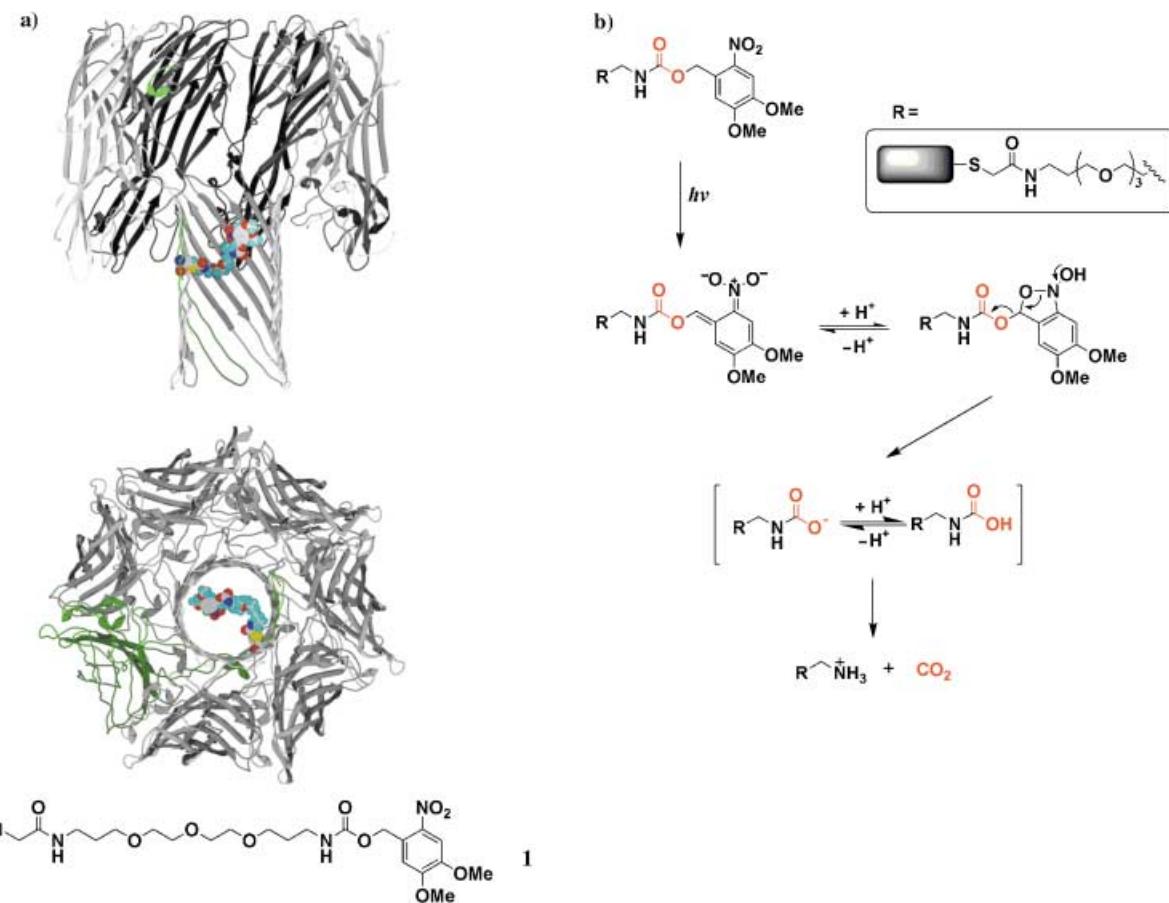


Figure 1. Single-molecule chemistry photoinitiated within an α -hemolysin pore. a) A model of the engineered α HL pore containing a single protected carbamate side chain is shown, which is based on the three-dimensional structure of the wild-type heptamer.^[28] The pore contains six unmodified subunits and one modified subunit. The latter is equipped with a 3,4-dimethoxy-6-nitrobenzylcarbamate group covalently attached through an oligo(ethylene glycol) linker to a cysteine residue at position 117 by derivatization with reagent 1. b) Possible photoinitiated chemistry. The most likely isomers and most abundant protonation states of the molecules are shown. A more detailed discussion can be found in the Supporting Information.

nitronate or the bicyclic intermediate (or possibly both species in rapid equilibrium; see Figure 1 b and the Supporting Information). The breakdown of similar nitronates, as monitored through their characteristic absorbance at wavelengths greater than 400 nm, occurs with rates that range from less than 1 s^{-1} to more than 10^5 s^{-1} .^[18] For example, $k = 1\text{ s}^{-1}$ for the 1-(3,4-dimethoxy-6-nitrophenyl)ethyl ester of glycine,^[19] while $k = 2 \times 10^5\text{ s}^{-1}$ for the α -carboxy-2-nitrobenzyl ester of glycine.^[20] The rate of breakdown of the nitronate in the present case is at the low end of the known range, with $k = 0.56\text{ s}^{-1}$ at pH 7.0. The rate increases at lower pH values, but the magnitude of the increase is only approximately fivefold over 4.5 pH units. Examples of weak pH dependence can be found in the literature,^[20,21] but the rate increase is more often three or four orders of magnitude.^[18]

The breakdown of carbamic acids have also been investigated in detail. Carbamates and related molecules are commonly used as protecting groups in organic synthesis and, under physiological conditions, lysyl residues in proteins are often carbamylated.^[22] The pH dependence of decarboxylation is striking and can be fit to Equation (1):^[23–25]

$$k_{\text{obs}} = \frac{k[\text{H}^+]}{K_a + [\text{H}^+]} \quad (1)$$

The value of K_a (approximately 10^{-5} M) is close to that of the ionization constant of carbamic acids ($\text{p}K_a$ ca. 5.2), which suggests that the rate of reaction is proportional to the concentration of the protonated species (RNHCOOH), although several studies suggest that the actual intermediate is the short-lived zwitterionic species (the concentration of which will be proportional to that of the protonated form).^[23,25] Our single-molecule data are in keeping with such an interpretation. The values for k_{obs} can be fit to Equation (1) to yield $k = 350\text{ s}^{-1}$ and $\text{p}K_a = 6.4$ (Figure 2e). The measured k' values at high pH values for carbamates of strong bases (where $k_{\text{obs}} = k'[\text{H}^+]$) are around $10^8\text{ M}^{-1}\text{s}^{-1}$; for example, $k' = 7.9 \times 10^7\text{ s}^{-1}$ for butylamine.^[23] Therefore, $k = k'K_a = 500\text{ M}^{-1}\text{s}^{-1}$, which is close to the value we find. Further experimentation will be required to determine how the shift in the apparent $\text{p}K_a$ value is related to the environment within the β barrel.

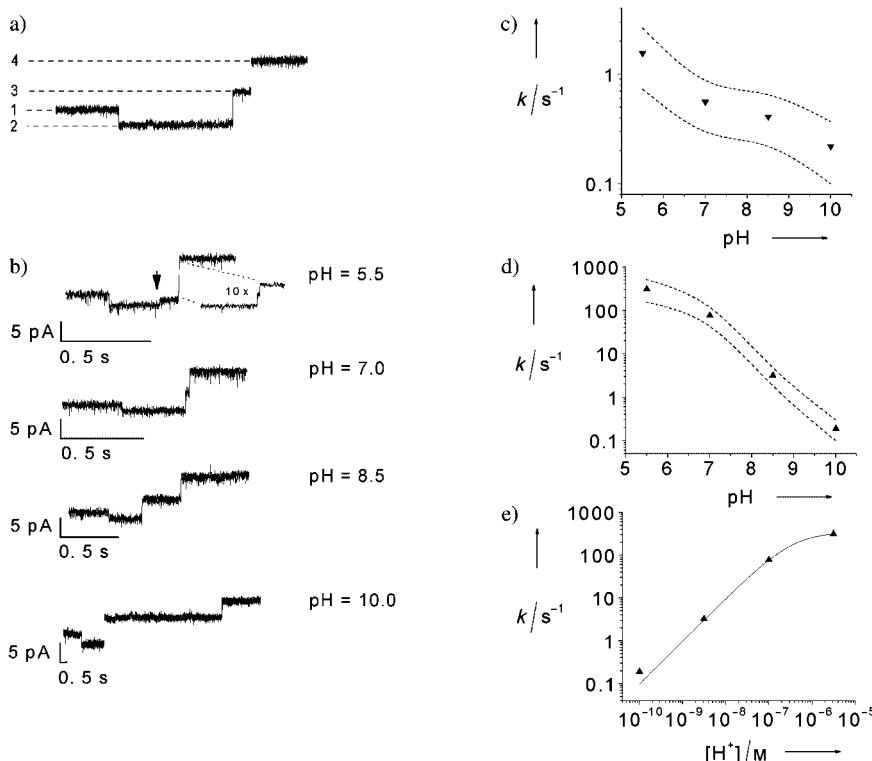


Figure 2. Single-molecule chemistry initiated by near-UV irradiation. a) Four levels in the single-channel current trace were observed. Level 1, before photon absorption, corresponds to the starting material. Level 2 is assumed to represent the nitronate intermediate (see Figure 1b). Level 3 is the carbamic acid as judged by the strong pH dependence of its breakdown. Level 4 is the protonated amine, the final product. Several studies suggest that the bicyclic intermediate (Figure 1b) is too short-lived^[29,30] to be observed under the recording conditions, which would reveal intermediates with $\tau \geq 1 \text{ ms}$. In some experiments, we saw small steps in the level 2 current (30% of recordings; for example, see Figure 2b, arrow), but their inconsistency makes it unlikely that they represent the bicyclic molecule. Of course, some intermediates might be electrically silent. b) Representative current traces showing the pH dependence of the reaction. The experiments described in this work were done in 2 M KCl, 10 mM tris(hydroxymethyl)aminomethane (Tris) base, 100 μM ethylenediamine tetraacetate (EDTA), titrated to the desired pH value with HCl. Similar results were obtained when 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer was used at pH 5.5 and 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) buffer at pH 10.0. c) Rate of nitronate breakdown plotted as a function of pH. The 90% confidence interval is shown (dashed lines). d) Rate of carbamic acid breakdown plotted as a function of pH. The 90% confidence interval is shown (dashed lines). e) The data in (d) are fitted to Equation (1) by using a nonlinear fitting algorithm (Origin 7.0) based on the Levenberg–Marquardt method for χ^2 reduction.

The determination of the kinetics of breakdown of the carbamic acid at low pH values exemplifies one of the advantages of using the single-molecule approach: the ability to measure the rate constant for a fast step that follows one or more slow steps. At pH 5.5, for example, the rate constant for the loss of CO_2 is $k = 313 \text{ s}^{-1}$, while the rate constant for the preceding step is $k = 1.6 \text{ s}^{-1}$. Therefore, in this case, the rate at which carbamic acid breaks down could not be observed by a more conventional approach such as flash photolysis of a bulk sample.

The β barrel of the α HL pore contains about 400 molecules of water. The present work and previous investigations of noncovalent interactions within the pore suggest that the properties of the solvent conform quite well with those of the bulk phase, which is in agreement with recent investigations

of confined water.^[26] Therefore, it should be possible to generalize the approach demonstrated here to the wide variety of chemistries that can be carried out in water^[27] and utilize many of the tools of mechanistic chemistry, such as the measurement of isotope effects and the trapping of intermediates.

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