Limitations of the "tethering" strategy for the detection of a weak noncovalent interaction†

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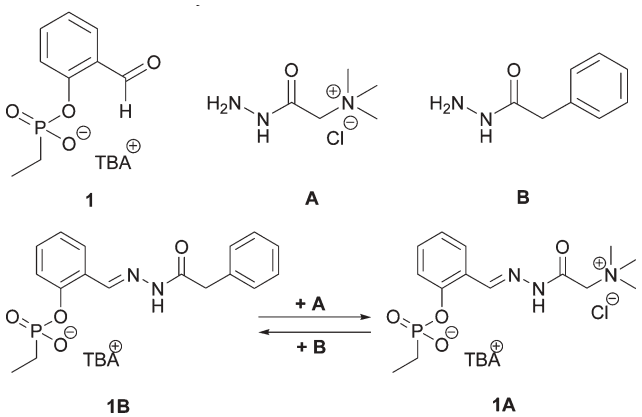
The success of applying the tethering strategy in a synthetic molecular system strongly depends on the experimental conditions and is related to the strength of the noncovalent interaction and the competition between the 'captured' and unbound ligand for the recognition site.

In 2000 Erlanson and co-workers reported a brilliant strategy called "tethering" to discover ligands from a library that bind weakly to targeted sites on a protein (thymidylate synthase) through covalent reversible disulfide bond formation.[1,2] Ligand selection without tethering would be impossible, because of the very weak affinity of the ligands for the protein. Since then, this strategy has been successfully applied for the selection of lead compounds for proteins such as IL-2,[3] PTP-1B,[4] and Tom20,[5] and for the detection of self-templating peptides.[6] Also synthetic systems are often characterized by weak noncovalent interactions, which means that, in principle, tethering should also be applicable for the selection of substrates for synthetic molecular receptors.[7] One has only to mix the library of putative substrates to the selected host and evaluate which conjugate is most dominantly present at the thermodynamic equilibrium.[8]

For this reason we decided to apply "tethering" for the selection of a molecule able to bind to a phosphonate (as a model of the transition state of a carboxylate ester hydrolysis) in a protic solvent where electrostatic and hydrogen-bonding interactions are relatively weak. After preliminary studies it became immediately evident that the selection process was strongly dependent on the conditions used and in some cases no selection at all could be observed. Intrigued by this behavior we decided to analyze a very simple system in order to clarify possible limitations of the "tethering" approach in synthetic systems and establish its conditions of validity.

Therefore we reacted 2-ethylphosphonoxybenzaldehyde **1** (2 mM) with an excess (3 equivalents each) of hydrazides **A** and **B** in MeOH-d_4 at 50 °C to form the corresponding hydrazones **1A** and **1B**, and let the mixture equilibrate to form the most stable product.‡ Our obvious prediction was that hydrazone **1A** would be the preferred product because of the intramolecular electrostatic interaction between the ammonium and the phosphonate group. This prediction was confirmed by the 70 : 30 ratio of **1A** : **1B** observed at thermodynamic equilibrium.§ That this amplification



was caused by the presence of the phosphonate group was evidenced by the fact that the identical experiment using 2-methoxybenzaldehyde instead of **1** yielded both hydrazones in equal amounts (see ESI†). However, when the competition experiment was repeated with an increasing amount of hydrazides **A** and **B**, the ratio between the two hydrazones **1A** and **1B** decreased to 59 : 41 when 25 equivalents of each hydrazide were present. The dependence of the ratio between the two hydrazones and the number of equivalents of hydrazides present is shown in Fig. 1a (■). The curve reaches a maximum when 5 equivalents of both **A** and **B** are added, but then the relative ratio between the two products diminishes following a trend towards the complete disappearance of the amplification.¶ Noteworthy, for the control compound 2-methoxybenzaldehyde in all cases a hydrazone ratio of 50 : 50 was found independent of the excess of hydrazide present (see ESI†).

An amplification in a dynamic combinatorial library which is dependent on the reaction conditions is highly undesirable.[9] In order to rationalize the observed trend in the formation of hydrazones **1A** and **1B**, we considered all possible equilibria present in solution (Scheme 1). Indeed one must consider not only the equilibrium between the two hydrazones **1A** and **1B** (K_{eq}), but also the possible interaction of the charged hydrazide **A** with each of them (defined by K_{AA} and K_{BA}, respectively).‖ Such an interaction competes with the stabilizing interaction between the phosphonate and the ammonium present in **1A**, and therefore, the resulting complexes **1A**·**A** and **1B**·**A** are expected to have very similar stabilities ($K_{eq,c} \approx 1$). Notably, assuming $K_{eq,c} = 1$, the equilibrium concentrations depend only on two parameters (K_{eq} and K_{BA}), since these define also the third equilibrium constant ($K_{AA} = K_{BA}/K_{eq}$). We implemented this model in a software program and the experimental points were fitted (Fig. 1(a), curve a).** The model describes the observed experimental data more

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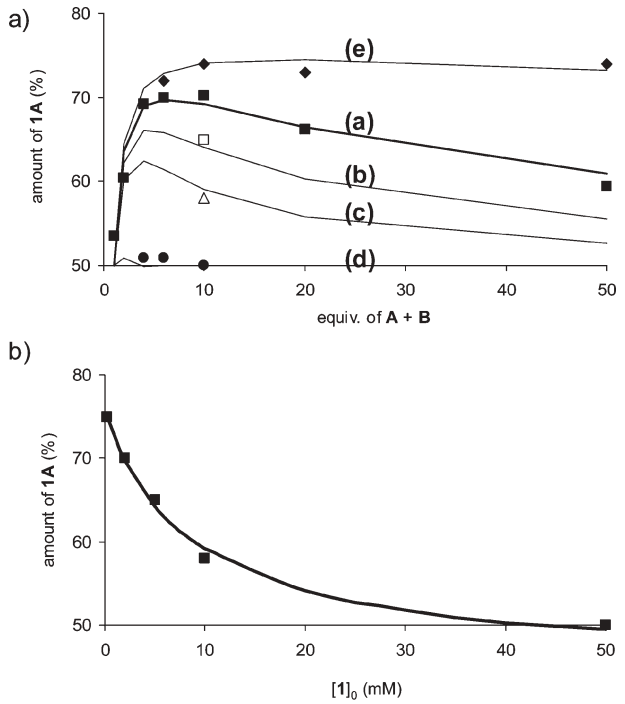
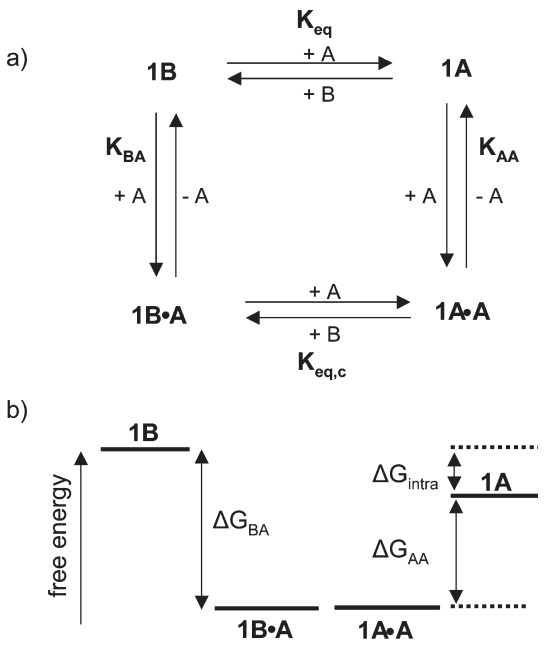


Fig. 1 (a) Amplification of **1A** as a function of the number of equivalents of hydrazides **A** and **B** present at various concentrations of **1** (◆: 0.2 mM; ■: 2 mM; □: 5 mM; △: 10 mM; ●: 50 mM). The solid lines represent either the obtained fit of the experimental data (a: [**1**] = 2 mM) or a simulation at different concentrations of [**1**] (b: [**1**] = 5 mM; c: [**1**] = 10 mM; d: [**1**] = 50 mM; e: [**1**] = 0.2 mM) using the model described in Scheme 1. (b) Observed amplification of **1A** as a function of the initial concentration of **1** in the presence of 5 equivalents of both **A** and **B**. The solid line represents the obtained fit using the model described in Scheme 1.



Scheme 1 (a) All equilibria taken into consideration to describe the amplification experiments.‖ (b) Representation of the relative thermodynamic stabilities of the species involved.

than satisfactorily, yielding values for K_{BA} and K_{eq} of 58 ($\pm$19) M^{-1} and 3.3 ($\pm$0.3), respectively. These values clearly illustrate that the system is highly effective in detecting very weak noncovalent interactions, but, more importantly, also shed light on the problems of applying the tethering approach to synthetic systems.

Upon adding increasing amounts of hydrazides, complexes **1A·A** and **1B·A** become the dominant species and a concomitant drop in amplification occurs because the difference in free energy between these complexes is smaller than that between **1A** and **1B**. Confirmation for this hypothesis was obtained by rerunning the competition experiment at higher initial concentrations of **1** (5 and 10 mM) adding 5 equivalents of each hydrazide. Higher concentrations favor the formation of the complexes **1A·A** and **1B·A**, and, in fact, a drop of the original ratio of **1A** : **1B** = 70 : 30 to values of 65 : 35 and 58 : 42, respectively, was observed (Fig. 1(a), points □ and △, respectively). In addition, these values nicely correlate with the calculated values based on model simulations (imposing the previously determined equilibrium constants for K_{eq}, K_{AA} and K_{BA}, curves b and c in Fig. 1(a)). Finally, no amplification at all was observed for an initial concentration **1** of 50 mM in the presence of either 4, 6 or 10 equivalents of **A** and **B**, which is consistent with the model predictions (Fig. 1(a), curve d).

While the values of equilibrium constants K_{AA} and K_{AB} can not be changed, the formation of complexes **1A·A** and **1B·A** can be suppressed by working under more diluted conditions. Accordingly, we have rerun the competition experiment using 10 times more diluted solutions monitoring the equilibration with HPLC (Fig. 1(a)). Unfortunately, working at dilute concentrations has the obvious drawback of slowing down exchange kinetics. In fact, for the samples containing up to 6 equivalents of hydrazide even heating at 50 °C for 1 week was not sufficient to reach the thermodynamic equilibrium. However, for the samples that contained more than 6 equivalents of hydrazide, the observed amplification of **1A** turned out to be constant up to the final sample containing 50 equivalents of hydrazide. A decrease in amplification was no longer detected. In addition, the observed maximum ratio for **1A** : **1B** of 75 : 25 is in excellent agreement with the calculated value of K_{eq} = 3.3 obtained from the NMR experiments (which corresponds to a maximum ratio of 76 : 24 for **1A** : **1B**). This value represents the maximum amplification that can be obtained for the combination of these building blocks. Fig. 1(b) summarizes the experiments described above. The relative amount of hydrazone **1A** is given as a function of the initial concentration of **1** in the presence of 10 equivalents of hydrazide. It clearly shows how the selection of the most stable compounds strongly depends on the initial concentration of molecular receptor **1**. The solid line represents the fit using the same model as described before, yielding nearly identical values for K_{BA} and K_{eq} (66 $\pm$ 8 M^{-1} and 3.5 $\pm$ 0.2, respectively).††

The optimum concentration that ensures the maximum amplification cannot be known *a priori* and this is, undoubtedly, a limitation of the "tethering" strategy. As a rule of thumb it is convenient to operate under high dilution conditions. These are very common conditions for the selection of targets for biomolecules,[1–6] but much less for synthetic molecular systems. However, dilute conditions might depress the reaction rate to such an extent that it may require incredibly long reaction times to

reach the thermodynamic equilibrium. This may pose limits to the type of reversible reaction used for "tethering". With this respect, it may be noteworthy that all successful (biological) applications of the "tethering" strategy[1–7] rely on disulfide formation. Disulfide formation is a fast reaction and very compatible with polar solvents. As a final comment, it should be pointed out that the above limitations of the "tethering" strategy are present regardless of the type of molecular receptor considered.

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Notes and references

‡ All new compounds were characterized by ^{1}H, ^{13}C and ^{31}P NMR spectroscopy, ESI-MS and HPLC. See ESI.†

§ The mixture composition was determined by integrating the respective signals for hydrazones **1A** and **1B** in the ^{1}H NMR spectrum. Throughout this study, the absence of any further changes in the ^{1}H NMR spectra upon standing at 50 °C was taken as an indicator that the thermodynamic equilibrium was reached. This was independently confirmed by a control experiment in which an identical ^{1}H NMR spectrum was obtained starting from two different mixtures (either preformed **1A** or preformed **1B**) upon standing at 50 °C (see ESI†).

¶ The observed amplification curve is not caused by a difference in ionic strength in the mixtures. In fact, the addition of an excess of tetramethylammonium chloride (TMACl, 100 mM) to mixture **1** : **A** : **B** = 2 : 12 : 12 mM caused only a minor decrease in the observed amplification (from 70 to 67%). In addition, rerunning the amplification experiment at constant ionic strength ([**A**] + TMACl = 50 mM) did not significantly affect the observed profile.

‖ The formation of other species (for example dimer **1A**·**1A** as the most likely candidate) that may affect the final product distribution cannot be ruled out. However, the ^{1}H NMR spectra of hydrazone **1A** recorded at 2 and 25 mM (see ESI†). were superimposable, showing no indication whatsoever of dimerization. Therefore, we decided to use a minimal model involving only the thermodynamic equilibria required to explain the experimental observations.

** The model was implemented in MicroMath Scientist for Windows, version 2.01. A detailed description is given in the ESI.†

†† At thermodynamic equilibrium the model gives a ratio of 49 : 51 for **1A** : **1B** instead of 50 : 50 when $[\mathbf{1}]_0$ = 50 mM. The reason is that the amount of hydrazide **A** involved in complex formation is subtracted from the amount of free **A**. Consequently, the slightly higher concentration of **B** shifts the equilibrium in favor of hydrazone **1B**.

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