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Authors: Xiujun Ling and Craig Wilcox

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A Molecular Torsion Balance Study: A Nearby Anionic Group Exerts Little Influence on Hydrophobic Interactions Between Nonpolar Surfaces

Xiujun Ling,[b] and Craig S. Wilcox*[a]

Abstract: Polar groups have a solvent ordering effect on water, and therefore may affect hydrophobic binding energies for nearby lipophilic surfaces. This would mean that determinations of excess surface free energy association energies require consideration of nearby polar functional groups. This paper reports results of a study to measure this possible effect. We conclude that in our models an anionic polar group nearby a hydrophobic surface has little or no effect on the magnitude of hydrophobic association.

The function and the shapes of receptors, enzymes, nucleic acids, lipids, drugs, and other biologically active molecules are ultimately determined by a balance among many small (less than 20 kJ/mol) energetic interactions.[1] These forces of interaction and equilibria are also influenced by solvation and cosolute effects. All of these influences have a characteristic time domain. In the majority of experiments important to health and technology the contributions of these component forces are difficult to measure accurately due to the myriad thermodynamic substates that represent bound and unbound states, folded and unfolded states, and solvation states.[2] For example, the evaluation of the interactions of amino acid side chains in a single protein are confounded by the freedom of motion within the protein. Accurate quantitative evaluation of these motions and their effect on protein function is beyond present theory and practice. Molecular torsion balances can provide less ambiguous and more precise data to evaluate individual side chain interactions, including interactions that contribute to hydrophobically assisted folding.

The concept of the molecular torsion balance was introduced 25 years ago when a torsion balance based on Tröger's base was used to quantify substituent effects on the CH-π interaction.[3,5] Tröger's base analogs have become widely used in supramolecular chemistry.[4] A second generation of molecular torsion balances based on Tröger's base featured a more symmetrical core structure and a hydrophilic glutaric acid side chain to improve water solubility.[6] This advance enabled the measurement of alkyl CH-π interactions in water, and also made it possible to investigate the effect of water on molecular folding when the lipophilic groups coming into contact were the approximate size of amino acid side chains, and demonstrated that a molecular torsion balance can be sensitive to such small scale hydrophobic effects. Briefly described, a molecular torsion balance experiment measures the equilibrium between two conformational states ('folded' and 'unfolded'). The molecular torsion balance is designed to isolate a defined intramolecular contact that is present in the 'folded' state but not present in the 'unfolded' state. The free energy change between the two states is defined as the free energy of folding, ΔG_{fold} (Figure 1).

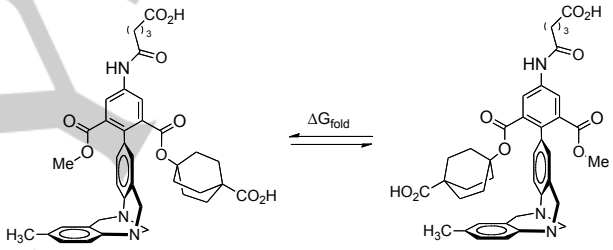

Figure 1. Illustration of free folding enersgy.

Gellman and Abbott examined the effect of cationic groups on hydrophobic association through AFM measurements and found that such groups do influence hydrophobic binding.[7] In this project we sought to use a molecular torsion balance to investigate whether or not polar and anionic groups nearby hydrophobic surfaces could have an effect on the magnitude of hydrophobically assisted folding. We refer to this as a "neighboring group effect" in hydrophobic surface association. We reasoned that this effect might arise from the influence of the polar group on the structure and orderliness of water at the hydrophobic surface. If the polar group influenced the excess free energy of the water at the nearby hydrophobic surface, a change in the contribution of desolvation to binding or folding energy might be observed.

[a] Prof. Craig. S. Wilcox
Department of Chemistry
University of Pittsburgh
Pittsburgh, PA 15260 USA
Fax: (+1) 412-624-1272
E-mail: daylite@pitt.edu
[b] Dr. Xiujun Ling
HM Health Solutions Inc.
Pittsburgh, PA 15222 USA
Fax: (+1) 412-680-1284
E-mail: xiujun.ling2014@gmail.com

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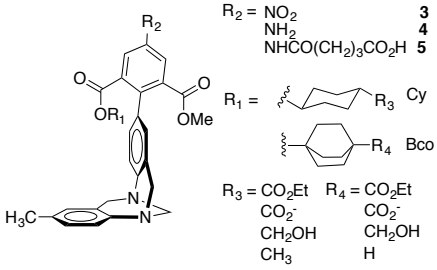


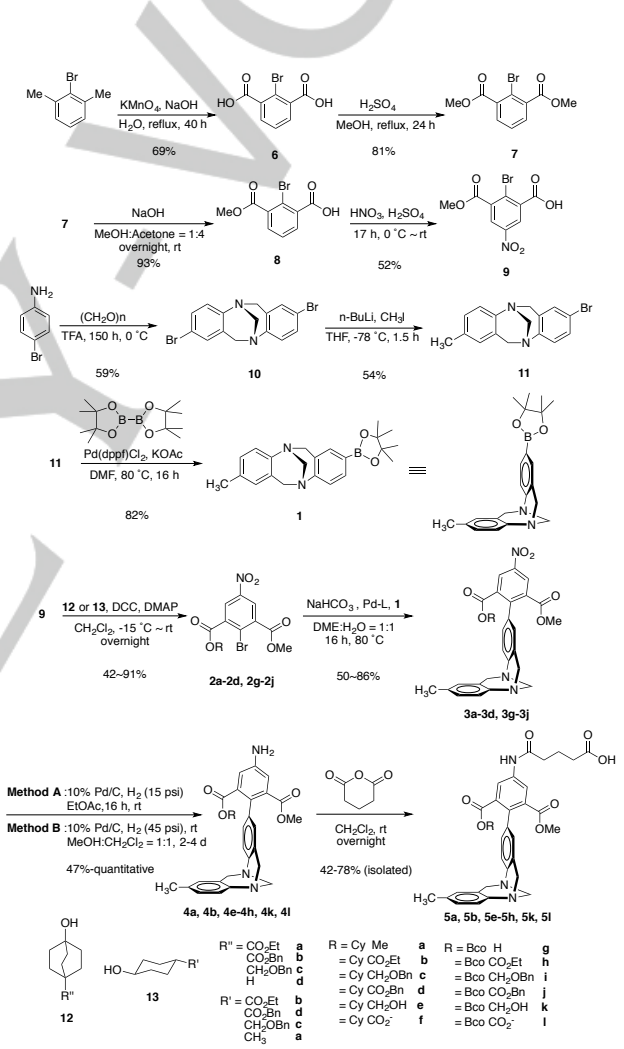
Figure 2. Cy and Bco series of torsion balances.

Several different molecular torsion balances were prepared to investigate these proposed neighboring group effects in hydrophobic binding. The water-soluble torsion balances in this paper incorporate a series of bicyclo[2.2.2]octane (Bco) and *trans*-cyclohexane (Cy) groups (Figure 2). The bicyclo[2.2.2]octyl alcohols used in the Bco series are symmetrical and rigid, and therefore reduce the number of thermodynamic substates that compose the folded and unfolded conformations, while the *trans*-cyclohexane (Cy) series provide an interesting comparison.

The synthesis of the Tröger's base core followed the method of Wärnmark.[8] The dibromo analog **10** was treated with n-butyl lithium and methyl iodide to give the 2-bromo-8-methyl analog **11**.[9] This product was transformed to the boronate analog **1** with catalytic Pd(dppf)Cl_2 (Scheme 1).[10,11] The nitro hemiester **9** was prepared in a sequence that started with the permanganate oxidation of m-bromoxylene.[12] Esterification of the resulting diacid **6** gave diester **7** as the product.[13,14] Diester **7** was then treated with 1.1 eq. of sodium hydroxide to yield hemiester **8**, which was transformed to nitro hemiester **9** by nitration with nitric acid and sulfuric acid. Hemiester **9** was converted to isophthalate esters **2a-d**, **g-j** in good yields through standard DCC esterification.[15] (Attempts to use EDC·HCl in the esterification reaction gave low yields with tertiary alcohols **12**.) Torsion balances **3a-3d** and **3g-3j** were synthesized by Suzuki coupling[16] of isophthalate **2** to Tröger's base **1**.[17] The reduction of the nitro group of torsion balances **3a**, **3b**, **3g** and **3h** was accomplished with 10% Pd/C and hydrogen at 15 psi. For those torsion balances with benzyl groups (**3c**, **3d**, **3i** and **3j**) reduction and debenzylation was accomplished with Pd/C at 45 psi of H_2. Acylation of the amino group on all homologs of **4** with glutaric anhydride afforded hydrophilic torsion balances **5**.

The synthesis of the bicyclo[2.2.2]octyl alcohol series **12** (Scheme 2) followed Grob and Rich's method.[18] It began with the reaction of diethyl acetoacetate and ethyl acrylate. The resulting triester **14** was hydrolyzed to generate diacid **15**, which then afforded ketoester **16** through decarboxylative cyclization.[19] Conversion to alcohol **17** was accomplished by a second cyclization. Deoxygenation of the carbonyl group on **18** was achieved with ethane dithiol and Raney Ni in two steps and afforded alcohol **12a**.[20] The synthesis of *trans*-cyclohexyl alcohols **13** and transformation of alcohol **13a** into **13b-13d** followed standard methods described in the supplementary information.[21]

The conformations of these molecular torsion balances were examined by analysis of the methyl ester signals in ^{1}H NMR spectra (Figure 3). The rotation around the single aryl-aryl bond is slow on the ^{1}H NMR time scale. When the methyl ester is on the concave side of the Tröger's base, the methyl protons resonate at 3.0 ppm. In contrast, when the methyl ester is on the convex side the methyl ester protons resonate at 3.7 ppm, as is typical for common methyl esters. This difference unambiguously identifies the folded and unfolded states.



Scheme 1. The synthesis of torsion balances **5**.

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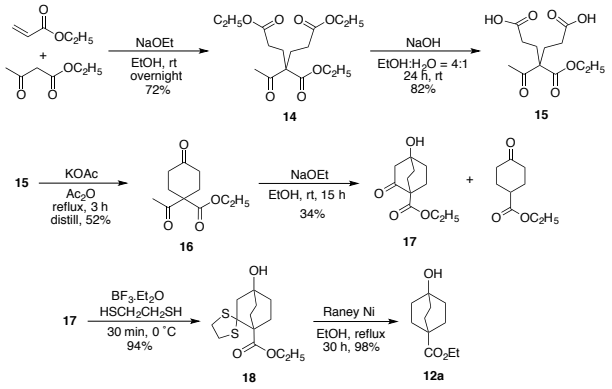

Scheme 2. The synthesis of bicycle[2.2.2]octyl alcohol **12a**.

In order to determine whether temperature would have an effect on the folding percentages of torsion balances, folding percentages of all torsion balances were measured at temperatures from 5 ˚C to 35 ˚C. This is a limited range due to the freezing point of the aqueous solution and the broadening of signals as temperature rises. In this range of temperatures folded and unfolded methyl ester resonance frequencies changed very little and the differences of folding percentages were within our bounds of error.[22]

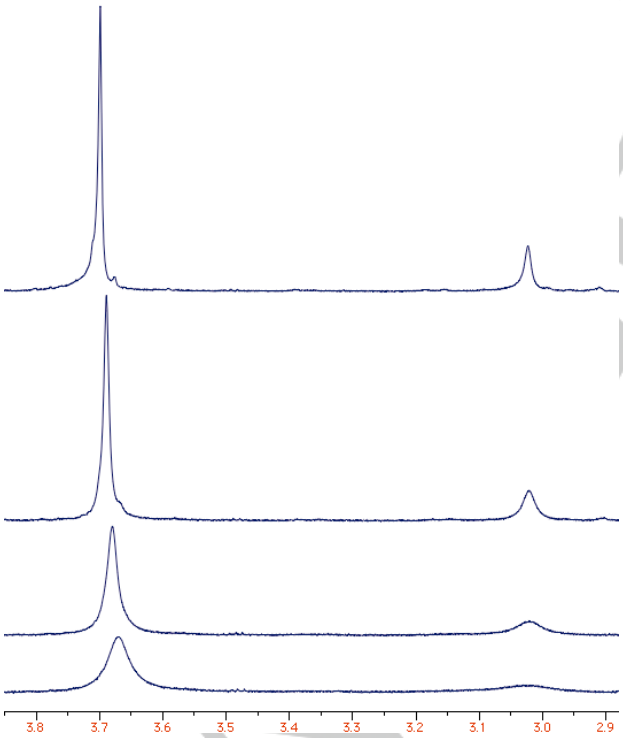

Figure 3. Methyl ester signals of torsion balance **3j** at 1 mM concentration in $CDCl_3$ at 5 ˚C, 15 ˚C, 25 ˚C and 35 ˚C (temperature increasing from top to bottom).

The torsion balances **5a**, **5b**, **5e**-**5h, 5k,** and **5l** were examined in two solvents (D_2O and CD_3OD) at 5 ˚C through 700 MHz NMR spectroscopy (Table 1). The folding energy in D_2O is significantly higher than it is in CD_3OD. This confirms again that a molecular torsion balance is sensitive to nanoscale hydrophobic effects.[6] The bicyclo[2.2.2]octyl series had higher folding energies than the cyclohexane series in both D_2O and CD_3OD. We think that this is because bicyclo[2.2.2]octyl has larger contact area than *trans*-cyclohexyl and lower rotational entropy in CD_3OD. The torsion balances bearing the ester or carboxylic group (entry 2 and 5) have higher folding percentages than the balances with hydroxyl or alkyl groups (entry 1 and 7) in the bicyclo[2.2.2]octyl series. This might be due to the branching nature of the carbonyl group allowing enhanced London dispersion interactions. In water, however, torsion balance **5g** (Table 1, entry 1) exhibited folding percentages similar to **5h** and **5l** (Table 1, entry 2 and 5). The ester and carboxylic groups have hydrogen-bonded interactions with water. If the exposed surface area of ester and carboxylic group in water is smaller than it is in unfolded state, the result would be a positive contribution to the folding free energies, making the free energies of **5h** and **5l** closer to **5g**. Torsion balances with a hydroxyl group **5e** and **5k** (Table 1, entry 7 and 8) are expected to form strong hydrogen bonds with water. Inhibition of hydroxyl group solvation in the folded state would decrease the percentage of conformers having the hydroxyl on the endo side (folded). Indeed, the data reveal diminished folding of these alcohols **5e** and **5k** (Table 1, entry 7 and 8) in water compared to their alkyl analogs **5a** and **5g** (Table 1, entries 1+3).

Table 1. Folding energy of torsion balances **5a**, **b**, **e-h**, **k**, **l** in D_2O and CD_3OD at 5 ˚C.[d,e]

Entry	R	Comp. #	ΔG_{fold}[b] CD_3OD kJ mol^{-1}	ΔG_{fold}[c] D_2O kJ mol^{-1}
1	-Bco-H	**5g**	-2.3	-4.2 [a]
2	-Bco-CO_2^-	**5l**	-3.3	-4.3 [a]
3	-Cy-CH_3	**5a**	-1.7	-3.4 [a], -3.1
4	-Cy-CO_2^-	**5f**	-2.3	-3.8 [a], -3.8
5	-Bco-CO_2Et	**5h**	-3.0	-4.2
6	-Cy-CO_2Et	**5b**	-1.9	-3.8
7	-Bco-CH_2OH	**5k**	-2.3	-3.5
8	-Cy-CH2OH	**5e**	-1.4	-3.0

[a] Sample basified with excess NH_4OH. [b] Indicates ±13.4% to ±8.1% relative error in free folding energy. [c] Indicates ±8.3% relative error in free folding energy. [d] Folding/unfolding ratios were determined from line shape

analysis[23] of the ^{1}H NMR spectra (700 MHz NMR). [e] Measurements were made on 1 mM solutions.

The maximum solubilities of torsion balances **5g** and **5l** (Table 1, entry 1 and 2) were less than 1mM in D_2O, and ^{1}H NMR signals were too weak to evaluate with line-shape analysis. Increased solubility was observed upon addition of NH_4OH. Folding of **5f** and **5a** (Table 1, entry 3 and 4) was unaffected by additions of NH_4OH. Comparisons of entries 1 and 2, and entries 3 and 4 reveal that the presence of the carboxylate nearby the interacting surfaces has no significant effect on the folding of these molecules.

The effects of pD on the folding energies of **5f** and **5l** were examined at 5 ˚C (Table 2). No significant change in folding energies were observed between pD 5.4 and 10.0. As expected, the solubilities of **5f** and **5l** decreased as the pD decreased. These observations are consistent with our initial observation that NH_4OH had negligible influence on the folding energy of these acidic molecules.

Table 2. The effect of pD on folding energy of torsion balance **5f** and **5l** in D_2O at 5 ˚C.[a,b]

Entry	pD	CO_2H-Cy-CO_2H ΔG_{fold} (1 mM) [c, d] **(5f)** kJ mol^{-1}	CO_2H-Bco-CO_2H ΔG_{fold} (1 mM) [c, d] **(5l)** kJ mol^{-1}
1	10	-4.1	-4.6
2	6.9	-3.8	-4.9
3	6.0	-4.0	-5.0
4	5.8	-3.9	-5.1
5	5.4	-4.0	-

[a] The concentrations of **5f** and **5l** were nominally 1 mM in all the experiments, but below pD=6 concentrations were limited by solubility. [b] All the NMR data in this table were determined from line shape analysis of the ^{1}H NMR spectra acquired at 700 MHz. [c] The error of ±8% in free folding energy is determined by the accuracy of the line-shape analysis.

The data show that the torsion balances bearing a carboxylic ester or carboxylic acid group in the distal position relevant to the ester attachment site have a higher folding energy than torsion balances with simpler groups in that position. We considered that this could be due to the branching nature of the ester/acid functional groups. The ester and acid, due to branching, would allow increased interaction with the methylated aromatic ring close to the ester alkyl group. In order to test this idea, a torsion balance bearing an isopropenyl group was designed and synthesized. The isopropenyl group is isosteric with a carboxylic acid, and similar to an ester in this context, but is less polar compared to an ester or a carboxylic acid.

Table 3. Folding free energy and folding percentage of torsion balances with branch functional groups at 5 ˚C. [b, c]

Entry	Name	Comp.#	ΔG_{fold} [d] kJ/mol	Solvent
1	NO_2-Bco-C(Me)=CH_2	**25**	-2.1	$CDCl_3$
2	NH_2-Bco-C(Me)=CH_2	**26**	-1.8	$CDCl_3$
3	CO_2H-Bco-C(Me)=CH_2	**27**	-3.0 [a]	CD_3OD
4	NO_2-Bco-CO_2Et	**3h**	-2.2	$CDCl_3$
5	NH_2-Bco-CO_2Et	**4h**	-2.2	$CDCl_3$
6	CO_2H-Bco-CO_2Et	**5h**	-3.0 [a]	CD_3OD
7	NO_2-Bco-CO_2Bn	**3j**	-2.4	$CDCl_3$
8	NH_2-Bco-CO_2H	**4l**	-1.6	$CDCl_3$
9	CO_2H-Bco-CO_2H	**5l**	-3.3 [a]	CD_3OD

[a] Examined in CD_3OD. [b] All the NMR data in this table were determined from line shape analysis of the ^{1}H NMR spectra acquired on a 700 MHz NMR. [c] All the torsion balances were examined at 1 mM concentration. [d] Indicates ±0.2 kJ/mol absolute error in folding free energy.

As shown in Table 3, the folding free energy of torsion balance **27** (entry 3) is similar to the folding energies of torsion balances **5h** and **5l** in CD_3OD (entry 6 and 9). Based on this observation, we conclude that the folding percentage of the torsion balance with esters in the distal position is mainly affected by the branching nature of the neighbouring group. The closer proximity of the branched group results in greater London dispersion attraction in the folded state. In this study we found no evidence that a nearby anionic polar functional group influences the free energy change of conformational change (folding) driven by hydrophobic effects. We note that it would be incorrect to consider this result to be in conflict with the important observations of Gellman and Abbott in their investigation of cationic group effects, which were acquired using a different technique (atomic force microscopy), on a surface at a solid-liquid interface.[7]

Conclusions

The results demonstrate a significant increase in folding for these molecules on changing the solvent from CD_3OD to D_2O. This is a measure of the greater ability of water (compared to methanol) to enforce solvophobic association. The folding of torsion balance **5l** was not significantly affected by changes in pD in the range of 10 to 5.4. Furthermore, the measured folding energies of **5g** (lacking a polar group) and **5l** (containing a nearby polar carboxylate group) in D_2O were within experimental error. Nearby hydroxyl groups also had insignificant effects on folding. The non-

polar isopropenyl group had an effect similar to other branched functional groups (carboxylic acid and ester). We conclude that in this context a polar group nearby a hydrophobic surface has little or no effect on the magnitude of hydrophobic association. The increase in folding energy in this set of experiments appears to be due to the branched nature of the functional group.

Abbreviations: DCC (N,N'-Dicyclohexylcarbodiimide); EDC·HCl (1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride); DMF (N,N-Dimethylformamide); DMAP (4-(Dimethylamino) pyridine); DME (Dimethoxyethane); Pd-L (Chloro(di-2-norbornylphosphino)(2'-dimethylamino-1,1'-biphenyl-2-yl)palladium(II)); $Pd(dppf)Cl_2$ ([1,1'-Bis(diphenylphosphino) ferrocene]dichloropalladium(II)).

Acknowledgements

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Keywords: torsion balance, folding energy, hydrophobic interaction, protein folding model.

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[21] The isolation of the *trans*-cyclohexyl alcohols from the *cis* was accomplished by column chromatography.

[22] The results of temperature effect on folding free energy can be found in the electronic supplementary information.

[23] Dynamic NMR calculations of simulated spectra were performed using iNMR® (http://www.inmr.net).

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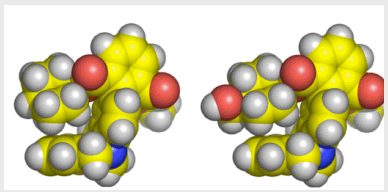
Minimized structures for truncated analogs of torsion balances **5g** and **5k**



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Layout 2:

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