

Thermodynamic Study of the Discrimination between Uridine and Thymidine Derivatives by Hydrophobic, Stacking, and Intercalating Interactions

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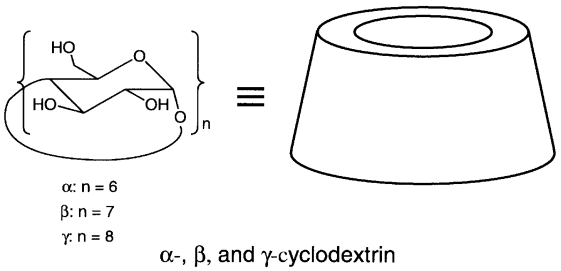
Thermodynamic parameters for the complexation reactions of uridine/thymidine nucleobases and related compounds, with hosts of differing binding modes and properties (natural cyclodextrins, 5,10,15,20-tetrakis (1-methlpyridinium-4-yl) porphyrin tetrachloride and bis-intercaland macrocycle) have been determined by titration microcalorimetry and/or fluorometry, in an aqueous buffer. For each of these hosts the effect of the 5-methyl group on the binding affinities was investigated. Although the affinities of uridine and thymidine towards cyclodextrins and 5,10,15,20-tetrakis(1-methylpyridinium-4-yl)porphyrin tetrachloride are very similar, the intercalation of these compounds into the bis-intercaland macrocycle has been shown to result in a high degree of discrimination of approximately 10 times. On the basis of thermodynamic data, the obtained contribution made by the 5-methyl group of thymidine to the structural characteristics of DNA and RNA is discussed.

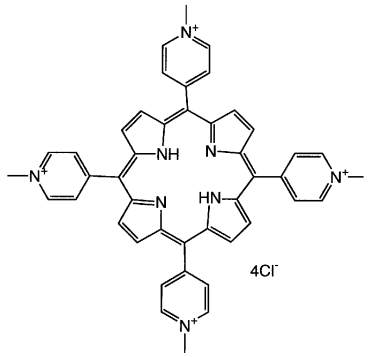
It is well known that the 5-methyl group of thymine plays an important role in the structure and biofunction of RNA and DNA macromolecules. For instance, the presence of thymidine's 5-methyl group changes the DNA's structural properties (dynamics, conformation, and stacking), and functions (nuclease accessibility and protein binding).[1–4] In double-stranded segments of tRNA, methylation and other modifications of the 5-position of uridine also play important structural roles and disrupt the formation of inappropriate conformations.[5–7] However, because of the complex nature of DNA and RNA, the explicit contribution made to the structure and function by the 5-methyl group has been difficult to assess.[8] In general, it is likely that the extra methyl group of thymine, compared to the lack of it in uracil, may have an impact on the structure in three ways: 1) due to additional hydrophobic interactions, 2) due to differences in the stacking properties, and 3) due to different intercalation characteristics. In this work we performed model studies of the effects of the 5-methyl group of thymidine compared with uridine, as well as a series of related guests on complexes made with three host molecules (Charts 1 and 2). Our main goal is not to mimic RNA and DNA in all possible details, but rather to use simple hosts to offset additional interactions which may complicate the thermodynamic behavior, and to reveal the "net" effect of hydrophobic, stacking and intercalation forces. Obviously, simple models cannot describe all of the structural complexity of native macromolecules. However, a similar thermodynamic behavior was found for various host–guest combinations including macromolecules if the same type of non-covalent forces are involved in the interaction (for instance hydrophobic interactions[9,10]); thus, the obtained experimental data may provide an insight into the thermodynamic driving forces of natural processes.

Natural cyclodextrins (CDs) were chosen as host compounds, because there are many similarities between the thermodynamic characteristics of cyclodextrin complexation reactions and typical hydrophobic interactions, e.g. molecular transfer from water to nonpolar organic media, which has been found experimentally.[9–15] The most probable mode of binding involves the insertion of the less polar part to the guest molecule into the cavity, whilst the more polar and often charged group of the guest is exposed to the bulk solvent outside the wider opening of the cavity. This picture is derived from both thermodynamic and NMR studies.[9b,11–13,16–21]

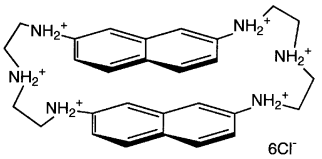
Hydrophobic interactions are not the only forces which are responsible for the stability of the cyclodextrin complex. Shape, size and the presence of various functional groups and their location also play an important role.[10,22,23] However, a number of papers have been published in which the authors claim that there is a correlation between the antagonistic or inhibitory activity of some compounds and the stability of their complexes with cyclodextrins.[24,25] Therefore, we may regard cyclodextrins as a good model of the hydrophobic pockets of biological macromolecules, where the hydrophobic interactions are the principal driving forces of complex formation. Porphyrins are a class of host molecules with an extended aromatic system, which facilitates complexation through stacking interactions.[26] 5,10,15,20-tetrakis(1-methylpyridinium-4-yl)-porphyrin tetrachloride was selected because of the presence of four positive charges, which might stabilize the stacked complex by an electrostatic interaction with the negatively charged guests. This also assists in a comparison of the thermodynamic parameters between the above-mentioned porphyrin with a bis-



α-, β, and γ-cyclodextrin



5,10,15,20-tetrakis(1-methylpyridinium-4-yl)porphyrin



bis-intercaland

Chart 1. Host molecules.

intercaland macrocycle, with both having the same charge under the applied experimental conditions. In the case of the bis-intercaland macrocycle[27] complexation occurs as a result of the intercalation of the aromatic ring of the guest molecule between the two aromatic rings of the host. By calorimetric experiments using these three representative host–guest systems, in which different kinds of interactions are the predominant contributors to the overall stabilization of the complex, we can assess how these discriminate between uridine and thymidine.

Experimental

Materials. Bis-intercaland macrocycle hydrochloride was synthesized[27] and provided by Lehn and Vigneron. The moisture content does not exceed 0.1%, judging from an elemental analysis.[27]

The purity of the bis-intercaland macrocycle was further investigated using capillary electrophoresis (Beckman PACE 5510 system, fused silica column (length = 57 cm, i.d. = 0.75 mm), electromotive force = 13 kV, sodium acetate buffer (0.015 mol dm^{-3}, pH = 5.03), and a diode array detector, wavelength = 200 to 600 nm). On the basis of the electrophoresis result, the mole-fraction purity of the bis-intercaland macrocycle is judged to be > 0.99.

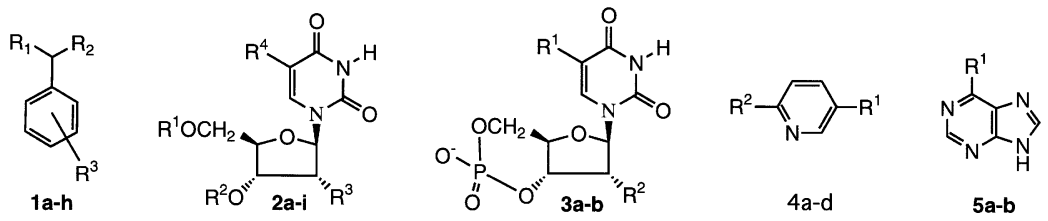
The samples of 5,10,15,20-tetrakis(1-methylpyridinium-4-yl)porphyrin tetrachloride used in this study were prepared by Porphyrin Products Inc. from their commercially available product, 5,10,15,20-tetrakis(1-methylpyridinium-4-yl)porphyrin tetratosylate, using ion-exchange columns. An evaluation of the water content was made based on an elemental analysis, and appropriate corrections to the initial concentrations of the 5,10,15,20-tetrakis(1-methylpyridinium-4-yl)porphyrin tetrachloride used in the microcalorimetric experiments were made for moisture content.

The stated purity of 5,10,15,20-tetrakis(1-methylpyridinium-4-yl)porphyrin tetrachloride based on a TLC analysis performed by Porphyrin Products Inc. (over silica gel eluted with a mixed solvent of 2-propanol, water, acetone, acetic acid, and concentrated aqueous ammonia in a ratio 3/3/1/2/1 by volume) was > 95%. It was also characterized by UV/vis. The purity of 5,10,15,20-tetrakis(1-methylpyridinium-4-yl)porphyrin tetrachloride was further investigated by our using capillary electrophoresis in a method similar to that described above for the bis-intercaland macrocycle. Two major peaks with relative areas of approximately 80% and 20% were found. The similarities in the UV/vis spectra of both peaks (±1.0 nm) and a consideration of the elution times in electrophoresis leads us to the conclusion that the second peak is 10,15,20-tris(1-methylpyridinium-4-yl)-5-(-4-pyridyl)-porphyrin with a charge of 3+. The implication of this is that the binding constants reported for complexes made with this host are at a lower limit for the fully *N*-methylated compound. For the reason that any ion-ion interaction made between the host and the guest has four-fold symmetry for the fully *N*-methylated compound, such interactions in the tri-*N*-methylated compound would be reduced by 25%. Therefore, statistically, for the bulk guest, the contribution of any ion–ion interactions to the observed binding constant would be 5% lower than that for the fully *N*-methylated compound. Taking into account that our main goal was to find the differences in the affinities between corresponding uridine and thymidine derivatives toward the host, it was not necessary to perform any further purification.

All other chemicals were purchased from Aldrich or Sigma and used without further purification. The purity of the compounds stated by the vendors was higher than 99% with the exception of 2′-deoxyuridine-5′-monophosphate disodium salt (98% by HPLC and spectroscopy), *N*-methyl-nicotinamide (97% by HPLC) and thymidine-5′-monophosphate sodium salt (98% by HPLC and spectroscopy). In addition to a purity analysis performed by the vendors, we have always performed Karl–Fisher determinations of the moisture content in the substances used, unless these data were available from the vendors.

Methods. The thermodynamic parameters were determined microcalorimetrically in various aqueous buffers at pH 4.8, 5.1, 6.9 or 10.0 to meet the usual requirements, $|pK_a(n) - pH| > 2$ and $|pK_a(n + 1) - pH| > 2$, since it was previously described[10–13] that the experimental results obtained by calorimetry are highly sensitive to the ambiguities of the host/guest protonation state. This issue is addressed in more details the subsection below (Complexation of uridine, thymidine, and related compounds with bis-intercaland macrocycle). The possible effect of a significant deviation from unity of the values of the activity coefficient (γ) of hosts, e.g. 5,10,15,20-tetrakis(1-methylpyridinium-4-yl)porphyrin or bis-intercaland macrocycle possessing a 4+ charge on the determination of equilibrium constants, is discussed subsection below (Complexation of uridine, thymidine, and related compounds with 5,10,15,20-tetrakis(1-methylpyridinium-4-yl)porphyrin).

The experimental and computational procedures for using a MicroCal ITC titration microcalorimeter were described previously.[11–13,28] Each microcalorimetric titration experiment consisted of 20 successive injections (5 μL per injection) of a buffer solution of



	R^1	R^2	R^3	R^4
1a: phenylacetic acid	CO_2^-	H	H	
1b: *o*-tolylacetic acid	CO_2^-	H	Me_{ortho}	
1c: *m*-tolylacetic acid	CO_2^-	H	Me_{meta}	
1d: *p*-tolylacetic acid	CO_2^-	H	Me_{para}	
1e: 3-phenylpropionic acid	$CH_2CO_2^-$	H	H	
1f: 3-phenylbutyric acid	$CH_2CO_2^-$	Me	H	
1g: 4-phenylbutyric acid	$(CH_2)_2CO_2^-$	H	H	
1h: 3-(*p*-tolyl)propionic acid	$CH_2CO_2^-$	H	Me_{para}	
2a: uridine	H	H	OH	H
2b: 2'-deoxyuridine	H	H	H	H
2c: 5-methyluridine	H	H	OH	Me
2d: uridine-5'-monophosphate(5'-UMP)	PO_3H^-	H	OH	H
2e: uridine-3'-monophosphate(3'-UMP)	H	PO_3H^-	OH	H
2f: uridine-2'-monophosphate(2'-UMP)	H	H	OPO_3H^-	H
2g: thymidine	H	H	H	Me
2h: thymidine-5'-monophosphate(3'-TMP)	PO_3H^-	H	H	Me
2i: thymidine-3'-monophosphate(5'-TMP)	H	PO_3H^-	H	Me
3a: uridine-3',5'-cyclic monophosphate(3',5'-cUMP)	H	OH		
3b: thymidine-3',5'-cyclic monophosphate(3',5'-cTMP)	Me	H		
4a: nicotinic acid	CO_2^-	H		
4b: nicotinamide	$CONH_2$	H		
4c: 6-methylnicotinamide	$CONH_2$	Me		
4d: *N*-methylnicotinamide	CONHMe	H		
5a: purine	H			
5b: 6-methylpurine	Me			

Chart 2. Guest molecules.

a guest into a reaction cell (1.36 mL) containing a host solution in the same buffer. An appropriate correction for the moisture content was made as described previously.[11–13,28] The uncertainties of the thermodynamic parameters presented in Tables 1 and 2 and mentioned in the text are two standard deviations of the mean, which were evaluated as described previously.[11–13,28]

It should be emphasized that in addition to calculations based on the 1:1 stoichiometric complex formation, we also performed calculations assuming $1:n$ and $n:1$ binding models whenever such higher-order complexes were suspected. However, such calculations did not lead to any appreciable improvement of the overall fit, and the assumption of a 1:1 model and a single binding site appears to be the only reasonable choice for all of the host-guest combinations examined.

Fluorometric titration experiments were performed at 25 °C in a conventional quartz cell at two different excitation wavelengths (300 and 322 nm), using a Hitachi F-4500 spectrofluorometer. The concentration of bis-intercaland was kept constant at 2×10^{-5} M (1M = 1 mol dm^{-3}), while the concentration of the guest was varied from 0.15 to 50 mM.

Table 1. Thermodynamics Parameters (K, ΔH)[a] for the Complexation Reactions of Various Aromatic Guests with 5,10,15,20-Tetrakis(1-methylpyridinium-4-yl)porphyrin tetrachloride at 298.15 K

Guest (charge)	Concentration/mM		pH	K/M^{-1}	ΔH/kJ mol^{-1}
	Guest	Porphyrin			
Phenylacetic acid (1−)	182	1.54	6.9[b]	34 ± 4	−8.8 ± 0.8
o-Tolylacetic acid (1−)	156	1.42	6.9[b]	84 ± 3	−8.9 ± 0.3
m-Tolylacetic acid (1−)	147	1.42	6.9[b]	64 ± 5	−9.0 ± 0.5
p-Tolylacetic acid (1−)	154	1.42	6.9[b]	50 ± 3	−11.8 ± 0.6
3-Phenylpropionic acid (1−)	183	2.17	6.9[b]	72 ± 2	−10.4 ± 0.2
3-Phenylbutyric acid (1−)	103	1.67	6.9[b]	40 ± 6	−6.3 ± 0.7
4-Phenylbutyric acid (1−)	98	1.67	6.9[b]	74 ± 6	−8.2 ± 0.4
3-(*p*-Tolyl)propionic acid (1−)	155	1.84	6.9[b]	97 ± 4	−12.7 ± 0.4
Benzoic acid (1−)	126	1.45	6.9[b]	67 ± 3	−16.4 ± 0.4
Nicotinic acid (1−)	185	2.17	6.9[b]	110 ± 4	−30.8 ± 0.7
Nicotinamide (0)	165	2.17	6.9[b]	56.6 ± 0.8	−41.4 ± 0.4
N-Methylnicotinamide (0)	149	1.26	6.9[b]	56.6 ± 1.0	−36.9 ± 0.5
6-Methylnicotinamide (0)	69	1.26	6.9[b]	103 ± 2	−44.2 ± 0.5
Purine (0)	153	1.54	6.9[b]	131.0 ± 0.6	−58.7 ± 0.2
Imidazole (0)	300	1.39	10.0[c]	5 ± 10	−6 ± 10
Tryptamine hydrochloride (1+)	73	0.92	6.9[b]	40 ± 2	−27.4 ± 1.0
Uridine (0)	156	1.50	6.9[b]	72.4 ± 1.0	−55.5 ± 0.4
Thymidine (0)	147	1.50	6.9[b]	100 ± 3	−54.9 ± 0.9
Uridine 3′,5′-cyclic monophosphate Sodium salt (1−)	97	1.51	6.9[b]	197 ± 3	−60.8 ± 0.9

a) Since the samples of 5,10,15,20-tetrakis(1-methylpyridinium-4-yl)porphyrin tetrachloride contain about 20% of 10,15,20-tris(1-methylpyridinium-4-yl)-5-(4-pyridyl)porphyrin impurities (see experimental section for more details), the equilibrium constants and reaction enthalpies derived from raw microcalorimetric data are the values which describe the particular samples involved. Consequently it is inappropriate to use such equilibrium constants for calculation of free energy and to define such reaction enthalpies as standard values, e.g. ΔH° or $\Delta H^{\circ\prime}$ even if it is reasonable to assume that bonding properties of the host with charge 4+ and 3+ is quite similar. b) Phosphate buffer: NaH_2PO_4 (0.025 M) + Na_2HPO_4 (0.025 M); pH = 6.9. c) Glycine buffer: $C_2H_5NO_2$ (0.1 M) + HCl; pH = 10.0.

Table 2. Thermodynamics Parameters (K, ΔG°, ΔH°, and ΔS°) for the Complexation Reactions of Various Aromatic Guests with Bis-intercaland Macrocycle (0.3 mM) at 298.15 K

Guest (charge)	Guests concn /mM	pH	K/M^{-1}	ΔH°/kJ mol^{-1}	ΔG°/kJ mol^{-1}	$T\Delta S^\circ$/kJ mol^{-1}
Uridine (0)	150–170	5.1[a]	34.2 ± 0.4	−50.4 ± 0.4	−8.76 ± 0.03	−41.6 ± 0.4
Thymidine (0)	120	5.1[a]	[c]			
5-Methyluridine (0)	150	5.1[a]	[c]			
2′-Deoxyuridine (0)	200–210	5.1[a]	22.9 ± 0.3	−51.5 ± 0.4	−7.76 ± 0.03	−43.7 ± 0.4
Nicotinamide (0)	235	5.1[a]	9.8 ± 0.6	−45 ± 2	−5.7 ± 0.2	−39 ± 2
6-Methylnicotinamide (0)	66	5.1[a]	12 ± 5	−60 ± 20	−6.2 ± 1.5	−54 ± 20
N-Methylnicotinamide (0)	190	5.1[a]	11.5 ± 1.0	−47 ± 3	−6.1 ± 0.2	−41 ± 3
Purine (0)	130	5.1[a]	72.2 ± 0.6	−45.6 ± 0.3	−10.61 ± 0.02	−35.0 ± 0.3
6-Methylpurine (0)	150	5.1[a]	97.3 ± 0.6	−45.4 ± 0.2	−11.35 ± 0.02	−34.1 ± 0.2
Uridine 3′,5′-cyclic monophosphate (1−)	60	5.1[a]	36.9 ± 1.5	−47 ± 2	−8.95 ± 0.10	−38 ± 2
Thymidine 3′,5′-cyclic monophosphate (1−)	80	5.1[a]	4 ± 3	−140 ± 100	−3 ± 3	−140 ± 100
Uridine 5′-monophosphate (predominantly 1−)[d]	40–80	5.1[a]	401 ± 5[e]	−45.3 ± 0.3[f]	−14.86 ± 0.03[g]	−30.4 ± 0.3[h]
Uridine 5′-monophosphate (predominantly 1−)[d]	60	4.8[b]	358 ± 6[e]	−44.9 ± 0.4[f]	−14.58 ± 0.04[g]	−30.3 ± 0.4[h]
2′-Deoxyuridine 5′-monophosphate (predominantly 1−)[d]	40	5.1[a]	303 ± 3[e]	−41.7 ± 0.3[f]	−14.16 ± 0.02[g]	−27.7 ± 0.3[h]
Thymidine 5′-monophosphate (predominantly 1−)[d]	180	5.1[a]	29.9 ± 0.8[e]	−62 ± 2[f]	−8.42 ± 0.06[g]	−54 ± 2[h]
Thymidine 5′-monophosphate (predominantly 1−)[d]	130	4.8[b]	26.0 ± 0.8[e]	−65 ± 2[f]	−8.08 ± 0.08[g]	−57 ± 2[h]
Uridine 3′-monophosphate (predominantly 1−)[d]	40	5.1[a]	165 ± 3[e]	−43.6 ± 0.7[f]	−12.66 ± 0.05[g]	−30.9 ± 0.7[h]
Thymidine 3′-monophosphate (predominantly 1−)[d]	150	5.1[a]	18.1 ± 0.6[e]	−67 ± 3[f]	−7.18 ± 0.08[g]	−60 ± 3[h]
Uridine 2′-monophosphate (predominantly 1−)[d]	50	5.1[a]	296 ± 4[e]	−42.5 ± 0.5[f]	−14.11 ± 0.04[g]	−28.4 ± 0.5[h]

a) Acetate buffer: $NaC_2H_3O_2$ (0.1 M) + $C_2H_4O_2$; pH = 5.1. b) Acetate buffer: $NaC_2H_3O_2$ (0.1 M) + $C_2H_4O_2$; pH = 4.8. c) K and/or ΔH° are too small to be determined by microcalorimetric method. d) Since pK_a of non-cyclic monophosphates were reported to be in the range of 6.3–6.5 (Ref. 34), approximately 95% of the species in the solution possess a 1− charge. e) Apparent equilibrium constant, since guest and probably the host-guest complex exist as a mixture of various ionic species in the solution at this particular pH. f) Under experimental conditions used ΔH_{cal} is equal to the $\Delta H^{\circ\prime}$ within the uncertainties reported (see text for details). g) $\Delta G^{\circ\prime}$ is determined from the apparent equilibrium constant. h) See text for the basis of determination of $\Delta S^{\circ\prime}$ values.

Results and Discussion

Complexation of Uridine, Thymidine, and Related Compounds with Natural Cyclodextrins. Uridine and thymidine exhibited very low affinity toward α-, β-, and γ-CDs. Only in the case of β-CD was noticeable complex formation with uridine ($K = 7 \pm 2$ M^{-1}, $\Delta H° = -7.6 \pm 1.0$ kJ mol^{-1}) and thymidine ($K = 10 \pm 3$ M^{-1}, $\Delta H° = -4.4 \pm 1.0$ kJ mol^{-1}) observed. This can simply be rationalized by greater size complementarity between uridine or thymidine and the β-CD. Reliable thermodynamic parameters cannot be derived from the results of microcalorimetric experiments in the cases of α- and γ-CDs, where the uncertainties exceed the thermodynamic parameters, themselves.

It should be mentioned that other neutral compounds possessing six-membered aliphatic or aromatic rings show much higher affinity toward natural CDs. For example, the equilibrium constant for the complexation of cyclohexanol[13] with β-CD is 704 M^{-1}, and that for the complexation of L-phenylalanineamide[28] with β-CD is 109 M^{-1}. The presence of the two oxygen and two nitrogen atoms in the aromatic rings of uridine and thymidine, leading to a reduced hydrophobicity of the six-membered rings in comparison with the other six-membered aliphatic or aromatic rings, is the likely reason for their very low affinity towards natural CDs. Consequently, the observed affinities of uridine and thymidine become comparable with highly hydrophilic carbohydrate guests, such as D-ribose.[29]

In our recent review,[10] we examined the effects of methylene increment for several different guest families upon the thermodynamic parameters. For almost all of such families, a steady enhancement of affinity towards α- and β-CDs (by a factor of 2–4) was observed. Uridine and thymidine have almost the same binding constant (within error) and, thus, are not effectively discriminated between upon complexation with CDs.

Complexation of Uridine, Thymidine, and Related Compounds with 5,10,15,20-Tetrakis(1-methylpyridinium-4-yl)porphyrin. Water-soluble porphyrins have attracted significant attention due to their ability to form complexes with neutral aromatic and negatively charged aromatic and aliphatic compounds in aqueous media.[26e,30–32] Furthermore, several artificial receptors based on porphyrins were synthesized for nucleotide recognition in aqueous media.[26d,26f] However there is only one example in the literature of benzoic acid and 3,5-dimetylbenzoic acid which allows us to explore the effect of methyl-derivatization to the aromatic ring on complex stability toward 5,10,15,20-tetrakis(1-alkylpyridinium-4-yl)porphyrin.[26e] The equilibrium constants were determined by UV/vis titration equal to 180 M^{-1} for benzoic acid and 210 M^{-1} for 3,5-dimetylbenzoic acid, indicating an insignificant effect of methyl-derivatization on the complex stability. However, such limited literature data does not allow a reliable generalization of the complexation behavior of guest molecules which differ by one methylene unit. For this reason, we performed microcalorimetric experiments with 17 different aromatic guest molecules in addition to uridine and thymidine (see Table 1).

The constant 4+ charge of the porphyrin host molecule at any pH allows us to use a wide range of pH values for microcalorimetric experiments. As we previously described,[11–13,28] the pH of the solution should be far from the pK_a of the guest and host to avoid possible co-existence of different ionic species in the reaction mixture, and/or protonation/deprotonation upon complexation. However, the activity coefficient (γ) of 5,10,15,20-tetrakis(1-methylpyridinium-4-yl)porphyrin possessing a 4+ charge could be far from unity in aqueous solution, even at a relatively low concentration of 1 mM. Thus, caution should be exercised in choosing guest molecules for microcalorimetric study. It is safe from a thermodynamic point of view to study complexation reactions with neutral guests, since such reactions are charge symmetrical with respect to the equilibrium equation. Furthermore, as described previously,[13] the activity coefficients of the free host (γ_H) and the host–guest (H·G) complex ($\gamma_{H\cdot G}$) compensate each other to a large extent in the equilibrium equation: $K = \gamma_{H\cdot G}[H\cdot G]/(\gamma_G[G]\cdot\gamma_H[H])$ (where, because the guest is neutral, unity is assumed for γ_G). As γ_G is close to unity for monoionic guest molecules at low concentrations (in the order of mM), we can assume it is also thermodynamically legitimate to compare the equilibrium constants derived from raw microcalorimetric data for a series of homologous monoionic guest compounds. Certainly, in such cases the reaction is not explicitly charge symmetrical, and thus there is a degree of uncertainty in claiming that γ_H and $\gamma_{H\cdot G}$ largely compensate each other in the equilibrium equation. Therefore, the equilibrium constants for such complexation reactions may have systematic deviation from their true value. Yet, we can assume that the ratio $\gamma_H/\gamma_{H\cdot G}$ should be reasonably constant for a series of monoionic guest molecules. Consequently, the activity coefficients compensate each other in the ratio of the equilibrium constants for a monoionic guest molecule and its methylated derivative. Thus, the data from Table 1 should be considered in terms of the differences between closely related guest molecules.

First of all, microcalorimetric experiments were used to investigate the effect of the addition to various guests of methyl groups around the aromatic ring on the binding to 5,10,15,20-tetrakis(1-methylpyridinium-4-yl)porphyrin, with this being the principal structural difference of interest between uridine and thymidine (Table 1). In comparison to phenylacetic acid, an increase in the affinity was observed for methylation at either the 2-, 3-, or 4-position (Table 1). This effect of the methyl group was also observed for 3-phenylpropionic acid with 3-(4-tolyl)propionic acid, and nicotinamide with 6-methylnicotinamide. In the comparison of uridine with thymidine we observed similar discrimination in binding affinity with the additional methyl group of thymidine resulting in a higher binding constant.

In contrast, when systematic addition of methylene groups to the guests was considered (and thus an increase in their overall hydrophobicity) in the series of phenylacetic acid, 3-phenylpropionic acid, 3-phenylbutyric acid, and 4-phenylbutyric acid, no correlation with the binding constants was observed. This highlights the differences in the binding properties between natural cyclodextrins and 5,10,15,20-tetrakis(1-methylpyridinium-4-yl)porphrin. Conversely, the increasing affinity

towards β-CD observed for the above series of compounds is in accordance with the increasing number of the guest's methylene units, and so the molecular hydrophobicity: $K = 17.4 \pm 2.4$ M^{-1} for phenylacetate,[11] 162 ± 4 M^{-1} for 3-phenylpropionate,[28] 415 ± 15 M^{-1} for 3-phenylbutyrate,[11] and 435 ± 35 M^{-1} for 4-phenylbutyrate.[11] It is difficult to separate the effects of ion-pairing and steric hindrance for above-mentioned guests in their complexation with 5,10,15,20-tetrakis(1-methylpyridinium-4-yl)porphyrin. However, it is clear that quite different steric effects are present for binding to β-CD and to 5,10,15,20-tetrakis(1-methylpyridinium-4-yl)porphyrin, because it is highlighted by phenyl ring methylation at the 2-, 3-, and 4-positions of phenylacetic acid. The size of the β-CD cavity allows the insertion of only the 3- and 4-substituted isomers, whereas methylation at the 2-position disrupts complexation.[11] In contrast, methylation at any position of the phenyl ring leads to an increase in the affinity for 5,10,15,20-tetrakis(1-methylphridinium-4-yl)porphyrin.

The effect of ionic interactions can also be observed in the doubling of the binding constant on going from nicotinamide to nicotinic acid. This enhancement of the affinity is not as large as the difference of ten times between benzoic acid and the derivative with two carboxy groups at the 1- and 4-position around the benzene ring, as reported by Schneider et al.[26e] It should be emphasized that our data and the data by Schneider et al.[26e] are not consistent with each other. In both experimental studies, similar qualitative trends due to methyl-derivatization, accompanying the size expansion of aromatic ring and the negative charge on the guest, were found. However, the obtained quantitative data are quite different from each other. For instance, the equilibrium constants for the complexation reaction of benzoic acid with 5,10,15,20-tetrakis(1-methylpyridinium-4-yl)porphrin differ by three times (see Table 1). In addition, neutral guests with similar sizes of aromatic rings, i.e. purine (in our study) and quinoline and quinoxaline (in study by Schneider et al.[26e]), exhibit five-times different affinity toward almost the same host. Concerning any of the above-mentioned disagreements, we cannot offer appropriate rationalization. We would like to mention that our methodology of microcalorimetric titrations[11–13,16,28] has been successfully applied for the precise determination of thermodynamic parameters of a wide variety of complexation reactions, a part of which is exemplified in Tables 1 and 2. We would also like to emphasize that the purity of the host (> 95%), determined by quantitative ^{1}H NMR analysis[26e] does not guarantee 95% molar purity of the porphyrin, since 5% of the insufficiently alkylated compound detected by NMR means the presence of 20% impurity (see, experimental section for details).

It should be emphasized that the reaction enthalpy is strongly dependent on the nature of the aromatic system involved in the complexation. For guest molecules possessing a phenyl ring, the reaction enthalpies vary from –8.2 to –12.7 kJ mol^{-1}. However, if the π-system is extended through carboxylate-phenyl conjugation (as present in benzoic acid and the nicotinic acid derivatives), more exothermic reaction enthalpies (from –16.4 to –44.2 kJ mol^{-1}) are observed, despite similar sizes.

The size of the guest's aromatic ring is also shown to be a significant factor. This principle is exemplified by a comparison of purine and imidazole (Table 1), with the much larger purine ring showing a very significant increase in binding affinity over imidazole. Indeed, if the aromatic ring of the guest molecule is large enough, even positively charged guests can interact with the host molecule. This was experimentally verified by the tryptamine cation, which showed appreciable affinity, comparable with neutral guests of smaller size.

Uridine and thymidine display slightly larger affinities towards the host than nicotinamide, but with slightly smaller affinities than purine, arising from the size of the interacting nucleobases, as discussed previously. The binding constants for complexation of uridine and thymidine are 72.5 M^{-1} and 100.0 M^{-1}, respectively. Again, the effect of the additional methyl group results in a higher binding affinity. However, it is likely that such a modest change in affinity would not be sufficient for effective discrimination between uridine and thymidine in biochemical reactions which involve porphyrin or other large planar aromatic systems as hosts.

Complexation of Uridine, Thymidine, and Related Compounds with Bis-intercaland Macrocycle. In contrast to 5,10,15,20-tetrakis(1-methylpyridinium-4-yl)porphyrin, which constantly possesses a 4+ charge in water, the bis-intercaland macrocycle possesses different charges at varying pH's. Its pK_a's were reported to be 2.50, 2.85, 7.39, 8.12, 8.85, and 9.47.[27] Thus, a solution pH of ca. 5 can satisfy the following requirements for the buffer solution used in the microcalorimetric experiments: $|pK_a(n) - pH| > 2$ and $|pK_a(n + 1) - pH| > 2$. In aqueous solution at pH 5, the bis-intercaland macrocycle exists predominantly as a cation with 4+ charge. Care should be exercised in interpreting the complexation thermodynamic parameters derived from raw microcalorimetric data, because the condition $|pK_a(n) - pH| > 2$ is practically impossible to meet at pH 5 for almost all carboxylic acids. For this reason the list of guest molecules studied was restricted to neutral guests and salts of orthophosphoric acid.

It should be mentioned that it was previously shown that the bis-intercaland host has broad binding abilities,[27] such as aliphatic and aromatic guests, along with the nucleotides AMP, GMP, CMP, and UMP. Here we considered the effect of methyl groups on the thermodynamics of intercalation by examining related guests differing by one methyl group.

Significantly, an inverted preference in affinity for uridine and thymidine was observed in comparison to the cyclodextrin and 5,10,15,20-tetrakis(1-methylpyridinium-4-yl)porphyrin hosts. Uridine exhibited appreciable affinity ($K = 34.2 \pm 0.4$ M^{-1}) toward bis-intercaland macrocycle, whereas thymidine showed no indication of complex formation. To further investigate the effect of methyl groups on intercalation into bis-intercaland macrocycle, 2′-deoxyuridine and 5-methyluridine were subjected to microcalorimetric analysis. Compared with uridine, 2′-deoxyuridine gave a reduced affinity by about 30%. However, no indication of complex formation was found for 5-methyluridine.

As was previously demonstrated, negatively charged guests

interact more strongly with a positively charged host than the corresponding neutral guests. In light of this, the affinities of the following cyclic monophosphate derivatives of uridine and thymidine towards bis-intercaland macrocycle were investigated: 3′,5′-cUMP and 3′,5′-cTMP, see Table 2. It is clear from these results that the addition of the monophosphate group does indeed result in a modest increase in the binding constants. This allows the ratio of discrimination between the uridine and thymidine derivatives to be determined, although with significant errors.

Because these cyclic monophosphates are conformationally restricted, an investigation of more flexible derivatives was undertaken, using 5′-UMP, 3′-UMP, 2′-UMP, 5′-TMP, and 3′-TMP as guests. It should be noted that under these experimental conditions these molecules can exist as either 1- or 2- anions; consequently, one may expect to observe higher binding affinities in comparison to 3′,5′-cUMP and 3′,5′-cTMP. However, the binding of the above non-cyclic monophosphates is likely to facilitate the release/absorption of protons upon complexation. Since dianions of the guests will exhibit higher affinity towards the macrocycle with a 4+ charge than monoanions, the ratio of mono- and dianions should shift in the direction of the dianionic species upon complexation. Thus, it should be realized that we are dealing with the calorimetric enthalpy of a reaction which contains the heats of several ionization reactions and, consequently, with apparent equilibrium constants. The significant pH-dependence of the K and ΔH_{cal} values obtained with these guests clearly indicates that these values would differ from the other data presented in Table 2 from a thermodynamic point of view. A determination of the entropy changes is only possible after an appropriate correction to the overall heat effect (ΔH_{cal}) is made, due to the heat effect of the interaction of released/absorbed protons with the buffer. The reason for choosing acetate buffer was that the correction is almost insignificant, since the ionization enthalpy of acetic acid is only -0.26 ± 0.08 kJ mol^{-1}.[33] Thus, even if one mole of protons is released/absorbed due to the formation of one mole of complex, the correction is comparable with, or even lower than, the reported uncertainties. Under such circumstances, it is thermodynamically acceptable to use raw microcalorimetric data for determining of entropy changes in the reactions. Certainly, in the present case the thermodynamic parameters determined from the apparent equilibrium constants should be denoted as $\Delta G^{\circ\prime}$, $\Delta H^{\circ\prime}$, and $\Delta S^{\circ\prime}$.

A comparison of the binding constants ratios for uridine/thymidine, 3′,5′-cUMP/3′,5′-cTMP (though with higher errors), 5′-UMP/5′-TMP, and 3′-UMP/3′-TMP reveals that the presence of a methyl group results in significant discrimination between uridine and thymidine containing guests molecules, with binding affinities differing by approximately an order of magnitude in all cases (Table 2). The small decrease in the affinity ($\approx$ 30%) from 5′-UMP to 2′-deoxy-5′-UMP (the similar behavior was discussed above for uridine and 2′-deoxy-uridine) is a confirmation that, indeed, 5-methyl is the main factor which determines the different thermodynamic behaviors of uridine and thymidine. It should be emphasized that despite the lower affinities observed for thymidine guests, larger exothermic enthalpies of complexation towards bis-intercaland are observed compared to the equivalent uridine guests. By no means can a differential entropy change $\Delta(T\Delta S^{\circ\prime})$ as large as the 24 ± 2 kJ mol^{-1} observed between 5′-UMP and 5′-TMP and $\Delta(T\Delta S^{\circ\prime})$ of 29 ± 3 kJ mol^{-1} between 3′-UMP and 3′-TMP (see Table 2) be assigned to the differences in solvation due to the presence of one methyl group. Even in the most "extreme" cases, entropy changes due to the solvation/desolvation of one methyl/methylene group do not exceed 4–5 kJ mol^{-1}, as exemplified in the literature.[9a,b;15a–c] Therefore, we conclude that complexes made with thymidine guests (as a result of the methyl group) are conformationally restricted, and thus exhibit the observed more unfavorable entropy changes compared to the corresponding uridine guests.

The apparent discrepancy between the stability constant reported for the complexation of 5′-UMP with bis-intercaland macrocycle[27] and that determined in this work is an important issue that will be addressed here. It should be noted that these two studies were performed in different buffers at different pHs, i.e. a pyridine–CF_3CO_2D buffer at pH 6 in the previous fluorometric study and an acetate buffer at pH 5.1 in this calorimetric study, thus the proton concentration differs by approximately ten times. Since pK_a of 5′-UMP was reported to be 6.4,[34] only about 5% of the guest molecules exist as dianions in the solution at pH 5.1, but at pH 6 this value increases up to 30–40%. The dianions of 5′-UMP can make one extra ion-pairing interaction with the host compared to the mono-anion. The affinity enhancement due to an extra ion-pairing interaction in water is expected to be ca. 5 times, as found for the complexation of aromatic diacids (2–) with specially designed cationic ligands (2+) that possessed two tetraalkylammonium residues at the correct positions to interact with the diacids.[35] In the present case, where the interaction is between a host of (4+) and a guest of (2–), a higher enhancement should be expected. Thus, at pH 6 there is about a one order-of-magnitude higher concentration of the dianionic species, which exhibit a higher affinity towards the tetracationic host by at least one order of magnitude in comparison to the monoanionic guest. Thus, it is reasonable to expect that the apparent equilibrium constant at pH 6 is larger by one order of magnitude (or even more) than that at pH 5. However, there are several examples in the literature that ionic interactions involved in complex formation can be masked by a steric hindrance or by other overwhelming interactions.[26e,36,37] The above discussion reveals the reason why in the present case a much larger value of the apparent equilibrium constant could be expected at pH 6 than at pH 5, indicating the need to perform fluorometric measurements under our experimental conditions (0.1 M acetate buffer at pH 5.1).

Fluorometric titrations were performed at two different excitation wavelengths, i.e. 300 and 322 nm. The excitation wavelength, 300 nm, used in a previous study[27] was found to be inadequate due to significant absorption by the 5′-UMP at this wavelength with higher concentrations. Thus, excitation at 322 nm was employed, since the guest has practically no absorption at this wavelength, even at high concentrations (up to 50 mM).

The original shape of the emission spectrum was very similar to that reported previously,[27] but the reduction of intensity arising from 5′-UMP addition was not as well pronounced as reported. The treatment of the raw experimental data by a nonlinear least-squares fitting gave an equilibrium constant of 580 ± 120 M^{-1} for excitation at 300 nm and 450 ± 80 M^{-1} for excitation at 322 nm. We conclude that the reduction of the equilibrium constant by 25% upon switching the excitation wavelength from 300 to 322 nm is assigned to the interference of the guest absorption at 300 nm. The fluorometrically obtained equilibrium constant (450 ± 80 M^{-1}) is in good agreement with that obtained from a microcalorimetric experiment (401 ± 5 M^{-1}). A result that lends further weight to the reliability of the thermodynamic data obtained by microcalorimetry is discussed here.

To further investigate the effect of the methyl group on intercalation, the thermodynamic parameters of binding to the bis-intercaland macrocycle were determined for purine, 6-methylpurine, nicotinamide, *N*-methylnicotinamide, and 6-methylnicotinamide (Table 2). An increase in the binding affinity is observed for 6-methylpurine compared to purine. *N*-Methylation in nicotinamide (in which the methyl group is distant from the aromatic ring) also results in an increase in the binding affinity. 6-Methylnicotinamide possesses direct methylation of the aromatic ring in a manner similar to the 5-methyl group of thymidine, and may thus be expected to have a corresponding reduction in affinity towards the bis-intercaland macrocycle. However, the experimental data reveal equal affinities for nicotinamide and 6-methylnicotinamide. Thus, uridine and thymidine are the only pair that exhibits high discrimination (about one order of magnitude), which arises from the presence of the 5-methyl group.

Conclusions

The affinities of uridine and thymidine towards cyclodextrins and 5,10,15,20-tetrakis(1-methylpyridinium-4-yl)porphyrin are very similar, and it is unlikely that these molecules are effectively differentiated between in biological systems by hydrophobic or "simple" stacking interactions. However, the complexation of these compounds with the bis-intercaland macrocycle has been shown to give high discrimination. Consequently, we can infer that the potential biological differentiation between uridine and thymidine can be achieved by intercalation, as clearly demonstrated in this study. Obviously, the thymidine molecule has a more restricted conformation when compared to the corresponding uridine molecule after complexation, as observed from the unfavorable entropy changes, which leads to the lower binding constant despite a more exothermic reaction enthalpy.

The induction of a more rigid conformation in DNA by the 5-methyl group would agree with the experimentally observed structural characteristics, such as the contribution to the stabilization of curved DNA sequences,[1,38,39] and to perturbations of the minor groove found in DNA upon removing the 5-methyl group from thymidine residues.[1] We thus see that the presence of thymidine in DNA (and the 5-methyluridine in double stranded tRNA[5–7]) may stabilize certain conformations, whilst destabilizing others, where the methyl groups would cause conformational restrictions with unfavorable entropy. Furthermore, it should be emphasized that in the RNA/DNA macromolecules unfavorable entropy changes arising from conformational restrictions caused by several 5-methyl groups would accumulate (since entropy is an intrinsically additive function), and thus the formation of certain conformations could be "entirely" thermodynamically prohibited.

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