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Transient kinetics and thermodynamics of anthroylouabain binding to Na/K-ATPase

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Abstract

The Na/K-ATPase is an integral membrane protein enzyme which uses energy derived from hydrolysis of ATP to pump Na^+ out of and K^+ into the cell. Ouabain belongs to a class of drugs known as cardiac glycosides, which are useful for treating congestive heart failure. Therapeutic value is achieved when these drugs bind to and inhibit the Na/K-ATPase of cardiac muscle. We gain insight into this important interaction by measuring the thermodynamics of the interaction of anthroylouabain (AO), a fluorescent derivative of ouabain, with the Na/K-ATPase. AO has the useful property that its fluorescence intensity is greatly enhanced ($\sim 10\times$) when it binds to the enzyme. Using this enhancement, we measure temperature dependence of transient kinetics for the association and dissociation of AO interacting with membrane fragments of Na/K-ATPase purified from dog kidney. Using a standard Eyring analysis, we find that the overall association of AO with the enzyme is driven by substantial contributions from both enthalpy and entropy, and that in an energy diagram for the association pathway, the free energy change is quite similar to that of ouabain deduced from previously published results [E. Erdmann, W. Schoner, BBA 307 (1973) 386]. However, in the transition state, there are substantial differences for the enthalpy and entropy, presumably due to the presence of the anthracene moiety.

Keywords: Anthroylouabain; Ouabain; Na/K-ATPase; Cardiac glycoside

1. Introduction

The Na/K-ATPase is an enzyme located in cell membranes which uses energy derived from hydrolysis of ATP to pump Na^+ out of and K^+ into the cell [1,2]. Ouabain belongs to a class of drugs known as cardiac glycosides, which are useful for treating congestive heart failure [3]. Therapeutic value is achieved when these drugs bind to and inhibit the Na/K-ATPase of cardiac muscle, which results in an increase in intracellular Na with a concomitant increase in intracellular Ca^{2+} due to decreased action of the Na/Ca exchanger. The higher levels of Ca^{2+} are conducive to stronger muscle contractions, which hopefully restore proper heart function. Thus, the interaction of cardiac glycosides with the Na/K-ATPase is of great importance. In this paper,

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we gain insight into this interaction by measuring the thermodynamics of the interaction of anthroylouabain (AO) [4], a fluorescent derivative of ouabain, with the Na/K-ATPase.

AO has been used in a variety of studies of the Na/K-ATPase, including investigations of: kinetics of cardiac glycoside binding [4,5], structure of and conformational changes of the enzyme [6–8], and levels of phosphorylated enzyme intermediates [9]. In addition to the desirable sensitivity and specificity provided by a fluorescent probe, AO has the additional useful property that its fluorescence intensity is greatly enhanced ($\sim 10\times$) when it binds to the enzyme [4]. It is this binding induced fluorescence enhancement that we make use of in this paper.

Kinetic studies and some thermodynamic studies of the interaction of ouabain with a variety of systems containing the Na/K-ATPase have been done using radioligand methods [10–15]. Generally, the kinetics are slow, with association rate constants on the order of 10^4 s^{-1} M^{-1} and dissociation rate constants on the order of 10^{-4} s^{-1} for interaction with the phosphoenzyme, resulting in highly specific binding with a dissociation equilibrium constant K_d around 10 nM. The kinetics of AO interaction with the Na/K-ATPase have been reported to be similar to those of ouabain [4,5,16].

In this paper, we measure the association and dissociation rate constants for the binding of AO to the phosphorylated Na/K-ATPase as a function of temperature in order to determine the thermodynamics of this interaction. We find that the rates for association and dissociation are relatively slow due to a high activation energy, and that the overall association of AO with the enzyme is driven by both entropy and enthalpy. These results are similar to the above mentioned results found for ouabain using radiolabelled methods. However, the transition state enthalpies and entropies for AO are quite different than for ouabain, suggesting that the presence of the anthracene moiety has a substantial effect on the binding process.

2. Materials and methods

Chemicals were from Sigma (St. Louis, MO). The buffer used for the experiments was 50 mM imidazole–HCl pH 7.0, 10 mM NaCl, 3 mM $MgCl_2$, 0.5 mM ATP, and 0.1 mM CDTA. This buffer causes phosphorylation of the enzyme. We use pH 7.0 as the standard pH in our laboratory's kinetic investigations of various steps in the Na/K-ATPase cycle. Enzyme concentration in the experiments was 0.2 μM. Frozen dog kidneys were obtained from Pel-Freez (Rogers, AR).

2.1. Enzyme purification

Na/K-ATPase was obtained using a modification of method C of Jørgensen [17], as described earlier [18]. Activities were measured using a coupled fluorometric assay [19]. The activity for the preparations was 6–7 μM Pi min^{-1} (mg enz)$^{-1}$ at 25°C.

2.2. Fluorescence measurements

AO fluorescence was measured on a fluorescence spectrophotometer (QM-1, PTI, Brunswick, NJ), with excitation at 367 nm and emission at 470 nm. Temperature was controlled with a circulating water bath. A stirring bar was run continuously in the cuvette.

Enzyme in the buffer was placed in a fluorescence cuvette in the spectrophotometer. Association of AO with the enzyme was measured by adding AO (typically 1 μM) to the cuvette and recording the subsequent fluorescence increase. Nonspecific association was measured by adding 100 μM ouabain to the phosphorylation buffer prior to addition of AO. Dissociation of AO from the enzyme was measured by adding 100 μM ouabain to the cuvette at the end of the AO association experiment (once the AO fluorescence achieved a constant value).

2.3. Mathematical model and data analysis

A standard bimolecular model with a stoichiometry of binding of 1:1 for the interaction of ouabain [11,13,14] or AO [4,5] with phosphorylated Na/K-ATPase has been previously established.

$$E + \mathrm{AO} \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} E - \mathrm{AO} \qquad (1)$$

The equilibrium dissociation constant is $K_d = k_{off}/k_{on}$. In an association experiment in which AO is added to the enzyme with the condition $[\mathrm{AO}]_{tot} \gg$

$[E]_{tot}$, the fluorescence enhancement which accompanies binding leads to the fluorescent signal [4,5]

$$F(t) = a + b(1 - \exp(-k_{obs}t)) \quad (2)$$

where

$$k_{obs} = k_{on}[AO] + k_{off} = k_{on}([AO] + K_d) \quad (3)$$

and a is the initial fluorescence and b is the magnitude of the fluorescence change.

The off-rate for AO dissociation is found by chasing off the bound AO through the addition of excess ouabain. When ouabain is at 100-fold excess over AO, the fluorescence decreases as

$$F(t) = a + b\exp(-k_{off}t) \quad (4)$$

where a and b are of the same values as in Eq. (2), but now a is the final fluorescence. The rate constants k_{on} and k_{off} were found by simultaneously fitting AO binding data and ouabain chase data to Eqs. (2) and (4).

Thermodynamic analysis was accomplished by standard Eyring transition state theory in which temperature dependence of the rate constants is given by

$$k = \left(\frac{k_b T}{h}\right)\exp\left(-\frac{\Delta G*}{RT}\right) = \left(\frac{k_b T}{h}\right)\exp\left(\frac{\Delta S*}{R}\right)\exp\left(-\frac{\Delta H*}{RT}\right) \quad (5)$$

where the $*$ refers to the transition state, k_b is the Boltzmann constant, and h is Planck's constant. If the transition entropy change $\Delta S*$ and the transition enthalpy change $\Delta H*$ are independent of temperature then they can be found by fitting Eq. (5) to the measured rate constants as a function of temperature. For the fitting, it is convenient to use the natural logarithm of Eq. (5),

$$\ln(k) = \ln\left(\frac{k_b}{h}\right) + \ln(T) + \frac{\Delta S*}{h} - \frac{\Delta H*}{R}\frac{1}{T} \quad (6)$$

Taking the ratio of the reverse and forward rate constants yields the van't Hoff equation

$$\ln(K_d) = \frac{\Delta H_0}{R}\frac{1}{T} - \frac{\Delta S_0}{R} = \frac{\Delta G_0}{RT} \quad (7)$$

which allows the change in enthalpy and entropy for the overall reaction, ΔH_0 and ΔS_0, to be found from the variation of the equilibrium binding constant with temperature. The standard state is 1 M of the enzyme and substrates.

Curve fitting was done using the Marquardt–Levenberg algorithm for nonlinear regression. Rate constants were found by simultaneously fitting Eqs. (2)–(4) to fluorescence data from association and chase experiments. Standard errors for the best fit values for the rate constants obtained from the curve fitting for individual experiments were less than 5% of the value. However, the fractional uncertainty due to variability in the best fit rate constants from repeated experiments was 15%. Uncertainty in the natural logarithm of the rates k_{on} and k_{off}, and their ratio, K_d, are then 0.15 and 0.20, respectively. Uncertainties in the transition state free energy changes, ΔG_f* and ΔG_r*, and the overall free energy change ΔG_0, found using Eqs. (5) and (7), are then 0.39 and 0.52 kJ mol^{-1}, respectively, which we rounded up to 0.4 and 0.5 kJ mol^{-1}. Uncertainties for ΔH and ΔS for the transitions and the overall association are the standard errors in best fit parameters resulting from curve fitting Eq. (6) and Eq. (7). Uncertainties for ΔH and $T\Delta S$ are in the range of 5–10 kJ mol^{-1}, which are much higher than the uncertainty in ΔG.

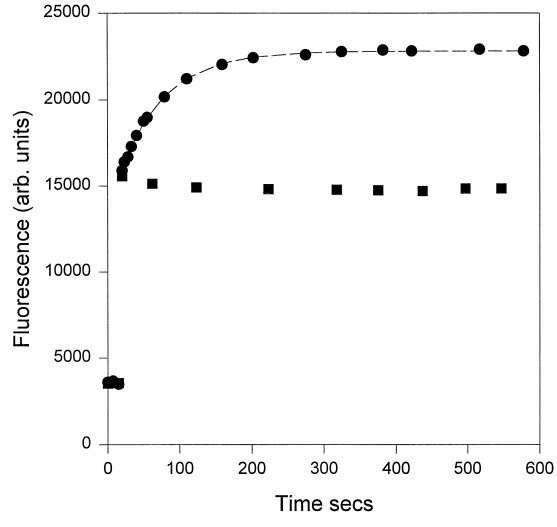


Fig. 1. Fluorescence when 1 μM anthroylouabain is added (at $t = 25$ s) to 0.2 μM Na/K-ATPase in the absence (●) and presence (■) of excess (100 μM) ouabain at 31°C. Also shown is the best fit to Eq. (2) (dashed line) giving an observed rate of 0.0163 s^{-1}.

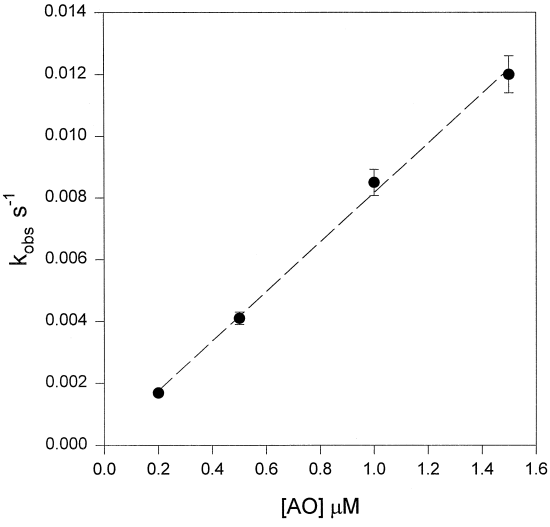


Fig. 2. Best fit observed rates for association of anthroylouabain with Na/K-ATPase at 25°C as a function of anthroylouabain concentration. Best fit to Eq. (3) (dashed line), giving $k_{on} = 8020$ s^{-1} M^{-1}, and $k_{off} = 1.6 \times 10^{-4}$ s^{-1}.

However, these uncertainties are coupled, since, the combination of enthalpy and entropy given by $\Delta H - T\Delta S = \Delta G$ must have the uncertainty of ΔG. This coupling is simply a result of the interdependence of the slope (ΔH) of a line and its y intercept (ΔS) in an Arrhenius type of plot when the line is constrained to pass near a point $(x = 1/310$ K, $y = \Delta G)$ off the y-axis.

3. Results

First, we verify that AO is binding at the ouabain binding site on the enzyme and we identify the fluorescence signal due to this binding. Fig. 1 shows the fluorescence when AO is added to a cuvette containing phosphorylated enzyme at 31°C. The circles show that upon addition of 1.0 μM AO, there is an initial rapid rise in fluorescence followed by a slower saturable increase. The squares show that the same initial rapid rise occurs when excess ouabain (100 μM) is present in the cuvette prior to the addition of AO; however, it is followed by a constant fluorescence instead of a slow saturable increase. When AO is added to a buffer containing no enzyme, there is an initial rapid rise followed by constant fluorescence which is nearly the same as for the 100 μM ouabain block. This demonstrates that the slow saturable increase in fluorescence can be attributed to specific binding of AO to the ouabain binding site of the enzyme and that the initial rapid increase in fluorescence is due to the fluorescence of free AO in the buffer. Further, any putative nonspecific binding of AO to the enzyme must be rapid and small in amount, and therefore does not affect the analysis of the specific binding. The dashed curve in Fig. 1 shows the best fit of Eq. (2) to the AO specific binding data. The best fit for the observed rate is 0.0163 s^{-1}.

The assumption of pseudo first order kinetics was checked by measuring the rate of AO association using different concentrations of AO. Fig. 2 shows that a plot of the best fit observed rates at 25°C versus AO concentration was well fit by Eq. (3), with a resultant slope of 8020 ± 280 s^{-1} M^{-1} for k_{on} and an intercept of $1.6 \times 10^{-4} \pm 2.6 \times 10^{-4}$ s^{-1} for k_{off}. The large uncertainty in the intercept just reflects the fact that measuring the association of AO with enzyme as a function of AO concentration is not a practical method for determining the dissociation rate constant. Determination of the dissociation rate constant is accomplished in the experiments

Table 1
Best fit kinetic parameters k_{on} and k_{off}, and their ratio, K_d, and the resulting free energies from Eqs. (5) and (7)

T (°C)	k_{on} s^{-1} M^{-1} ± 15%	ΔG_f * ± 0.4 (kJ mol^{-1})	k_{off} s^{-1} ± 15%	ΔG_r * ± 0.4 (kJ mol^{-1})	K_d nM ± 20%	ΔG_0 ± 0.5 (kJ mol^{-1})
20	2900	52.3	4.0×10^{-5}	96.4	13.8	−44.1
25	8200	50.7	1.2×10^{-4}	95.4	14.6	−44.7
31	15,100	50.2	3.0×10^{-4}	95.0	19.9	−44.8
37	39,000	48.8	7.95×10^{-4}	94.4	20.4	−45.6
42	93,500	47.3	2.05×10^{-3}	93.5	21.9	−46.2

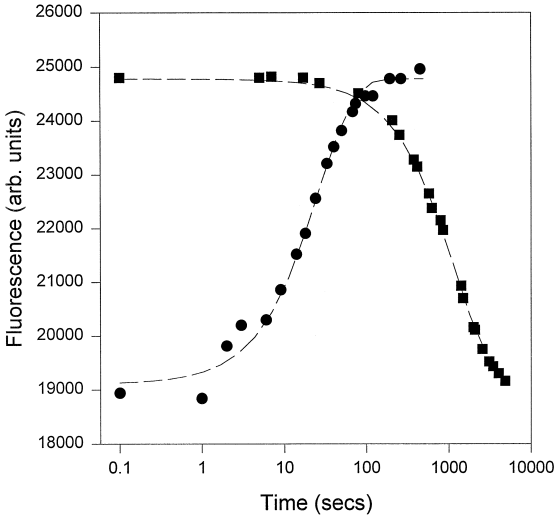


Fig. 3. Fluorescences from anthroylouabain association experiment (●) and its subsequent ouabain chase (■). Dashed line shows simultaneous best fit to Eqs. (2) and (4). Note that the time axis is logarithmic and that the chase has been shifted so both have the same time (zero).

where the AO is chased off by addition of an excess of ouabain. The observed rates in Fig. 2 were all measured using the same enzyme preparation and stock solutions, resulting in smaller percent uncertainties than for rates in Table 1 which were the result of experiments using different enzyme preparations and stock solutions. We also point out that although Eqs. (2) and (3) are strictly valid only when $[AO]_{tot} \gg [E]_{tot}$, we show in Appendix A that the expected error in the retrieved value of k_{on} for the range of [AO] we use is under 2%.

Fig. 3 shows the AO fluorescence signal due to specific binding for both the AO association and the subsequent AO chase with excess ouabain at 37°C. One micromolal AO was added to 0.2 μM phosphorylated enzyme. After the fluorescence reached a steady value, the AO was chased off by the addition of excess (100 μM) ouabain. Also shown is the simultaneous best fit to Eqs. (2) and (4). The resulting values for the rate constants for this particular trial are $k_{on} = 37{,}400\ s^{-1}\ M^{-1}$ and $k_{off} = 8.3 \times 10^{-4}\ s^{-1}$. Note that the time axis is logarithmic and that the chase was shifted so that the addition of excess ouabain occurred at time zero.

The observed off-rate in the chase experiments was not affected when the concentration of ouabain was doubled from 100 to 200 μM. This verifies that the fluorescence decay in the chase experiments was rate limited by the dissociation of the AO, so that Eq. (4) is appropriate.

Table 1 gives the temperature dependence of the best fit values for the rate constants k_{on} and k_{off}, and their ratio K_d, obtained as described in Section 2 by fitting Eqs. (2) and (4) to fluorescence data from the

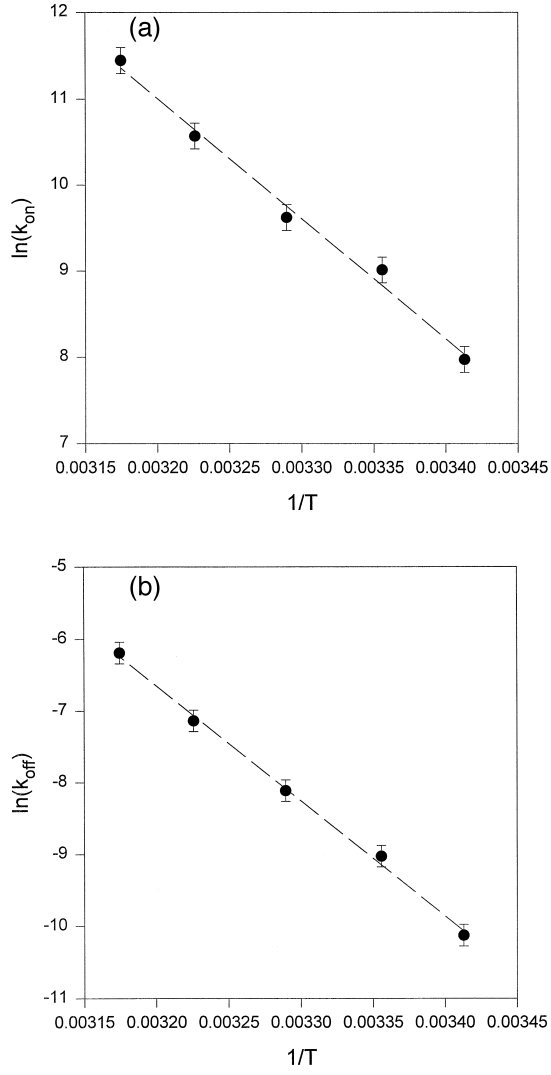


Fig. 4. (a) ln (k_{on}) and (b) ln (k_{off}) versus $1/T$, and best fits to Eq. (6) resulting in parameters for transition state enthalpies and entropies for the interaction of anthroylouabain with the Na/K-ATPase.

Table 2
Best fit parameters for enthalpies and entropies from Figs. 4 and 5 using Eqs. (6) and (7)

	ΔH [kJ mol^{-1}]	ΔS [kJ (mol K)$^{-1}$]	$-(310\ \mathrm{K})\,\Delta S$ [kJ mol^{-1}]
Forward	113.7 ± 10.5	0.210 ± 0.035	-65 ± 11
Reverse	131.1 ± 7	0.119 ± 0.025	-37 ± 8
Overall	-17.4 ± 5.5	0.091 ± 0.02	-28 ± 6

As explained in the text, the uncertainties in ΔH and ΔS are coupled so as to satisfy the much smaller uncertainties in ΔG shown in Table 1.

additions of 1 μM AO to 0.2 μM enzyme followed by 100 μM ouabain chase. Also shown are the transition state free energy changes, $\Delta G_{\mathrm{f}}*$ and $\Delta G_{\mathrm{r}}*$, for the forward and reverse direction found using Eq. (5), and the free energy change for the overall association, ΔG_0, found using Eq. (7). The uncertainties are due to variability in the best fit kinetic parameters from different experiments as described in Section 2.

Fig. 4 shows the plots of ln (k_{on}) and ln (k_{off}) versus $1/T$ along with the best fits to Eq. (6). The best fit values for the transition state enthalpies, $\Delta H*$, and entropies, $\Delta S*$, and uncertainties are in Table 2. Note that these uncertainties are much larger than those for the transition state free energies shown in Table 1; however, as described in Section 2, they are coupled such that $\Delta H* - T\Delta S* = \Delta G*$ satisfies the much smaller uncertainty of $\Delta G*$.

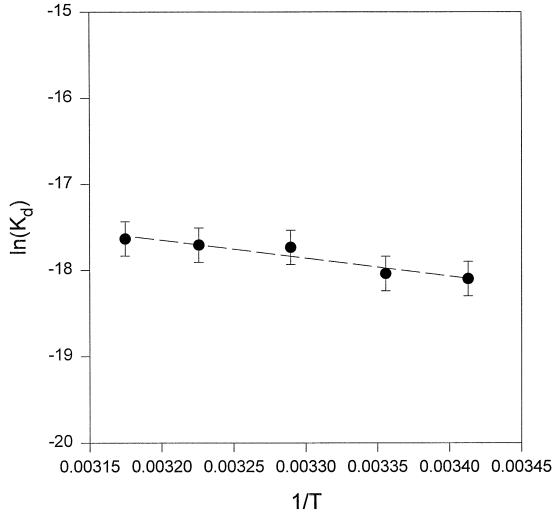
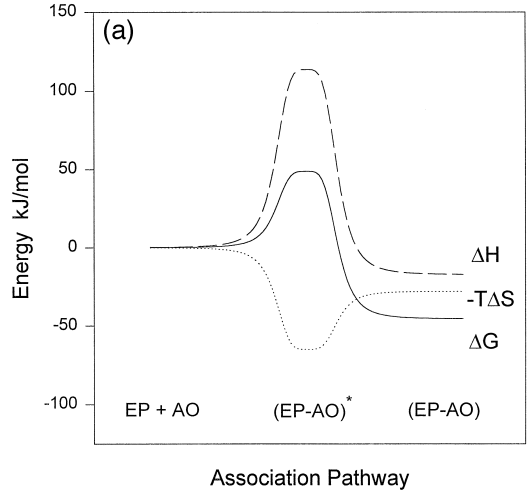


Fig. 5. ln (K_{d}) versus $1/T$, and van't Hoff plot of best fit to Eq. (7) resulting in parameters for enthalpies and entropies for the overall association of anthroylouabain with the Na/K-ATPase.



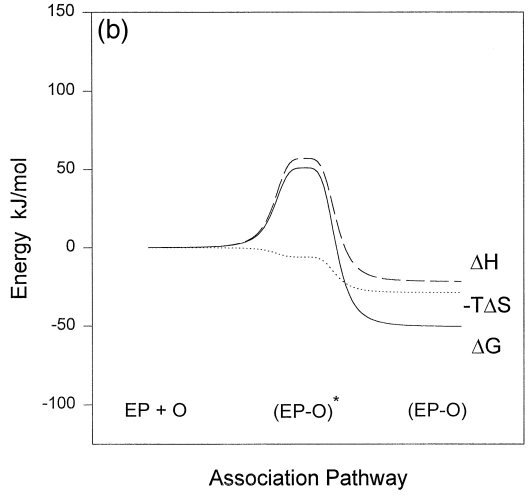


Fig. 6. (a) Energy diagram for the association of anthroylouabain (AO) with phosphorylated Na/K-ATPase (EP) constructed using thermodynamic parameters in Table 1Table 2. (b) Energy diagram for the association of ouabain (O) with phosphorylated Na/K-ATPase (EP) constructed from the kinetic data in the work of Erdmann and Schoner [11] by applying the same analysis that was used on our anthroylouabain kinetic data.

Fig. 5 shows the van't Hoff plot using the equilibrium dissociation constant determined by the rate constants in Table 1 and the best fit to Eq. (7). The resultant enthalpies and entropies for the overall reaction are shown in Table 2. As with the transition state energies, these uncertainties are coupled so as

to satisfy the much smaller uncertainty in ΔG_0 shown in Table 1.

Fig. 6a shows the resultant energy diagram for the association of AO with the enzyme, constructed using the thermodynamic parameters in Tables 1 and 2.

4. Discussion

Cardiac glycosides are useful drugs for treating congestive heart failure because of their ability to bind to and inhibit the Na/K-ATPase. We investigated this interaction by conducting a thermodynamic analysis of the interaction between the Na/K-ATPase and anthroylouabain (AO), a fluorescent version of the cardiac glycoside ouabain. Transient kinetic measurements were made to find both the forward and reverse rate constants as a function of temperature. The interaction was well fit by a model with a single homogeneous binding site. A standard Eyring analysis was used to find the thermodynamic parameters for the transition state as well as the overall association. An insight about the binding process is provided by the resulting energy diagram shown in Fig. 6a.

The kinetics of association and dissociation shown in Table 1 and the resulting tight specific binding with a K_d of 15–20 nM determine the free energy barriers of 50 and 95 kJ mol^{-1} for association and dissociation, respectively, and the overall free energy change of -45 kJ mol^{-1} shown in Fig. 6a. These results are consistent with the kinetic and equilibrium results for AO binding to Na/K-ATPase from eel [5,16] which found relatively slow kinetics, $k_{on} = 15{,}000\ s^{-1}\ M^{-1}$ and $k_{off} = 1.7 \times 10^{-4}\ s^{-1}$ at 25°C, and a high affinity of 3 to 12 nM depending on conditions.

Table 2 and Fig. 6a also show the changes in enthalpy and entropy which combine to give the resultant free energy change for the binding process. Note that the overall association of AO with phosphorylated Na/K-ATPase is driven by both entropy and enthalpy. As described in Section 2, the uncertainties in ΔH_0 and ΔS_0 are high compared to the uncertainty in ΔG_0, but they are coupled, since, their combination, $\Delta H_0 - T\Delta S_0 = \Delta G_0$, is required to have the much smaller uncertainty of ΔG_0. Therefore, the possible combinations of ΔH_0 and $-T\Delta S_0$ at 37°C range from -12 and -34 kJ mol^{-1}, respectively, to -23 and -22 kJ mol^{-1}, respectively, so that their sum is close to -45.5 kJ mol^{-1}, the value of ΔG_0 shown in Table 1. In any case, the association of AO with the Na/K-ATPase is driven by both enthalpy and entropy.

For the transition state, the enthalpy barriers $\Delta H*$ can be equated to activation energies in an Arrhenius analysis. The activation energy is 114 kJ mol^{-1} for the forward direction, and 131 kJ mol^{-1} for the reverse direction. These high activation energies are partially compensated for by the increased entropy of the transition state. As was the case for the overall interaction, the uncertainties for $\Delta H*$ and $\Delta S*$ for the forward and reverse directions to the transition state are coupled, so the combination $\Delta H* - T\Delta S* = \Delta G*$ has the smaller uncertainty of $\Delta G*$ shown in Table 1. For the forward direction, the possible combinations of ΔH_f* and $-T\Delta S_f*$ at 37°C range from 103 and -54 kJ mol^{-1}, respectively, to 124 and -76 kJ mol^{-1}, respectively, so that the combination is close to 49 kJ mol^{-1}, the value of ΔG_f* shown in Table 1. For the reverse direction, the possible combinations of ΔH_r* and $-T\Delta S_r*$ at 37°C range from 124 and -29 kJ mol^{-1}, respectively, to 138 and -45 kJ mol^{-1}, respectively, so that the combination is close to 94 kJ mol^{-1}, the value of ΔG_r* shown in Table 1.

It is of interest to compare our thermodynamic results for AO found using fluorescence methods with those of Erdmann and Schoner [11] for ouabain found using radiolabelled methods. They give forward and reverse rate constants, and thermodynamic parameters for the overall association of ouabain with the enzyme (Fig. 6 of Ref. [11]). For comparison purposes, we performed the same analysis on their data as we used on the data from our AO binding experiments. The resulting association pathway is shown in Fig. 6b. Comparison of Fig. 6a and b shows that the free energy change for the binding process is quite similar for ouabain and AO. This similarity is expected since the kinetics and equilibrium constants for ouabain and AO have been shown to be similar [5]. In both cases, the free energies of activation for the forward (~ 50 kJ mol^{-1}) and reverse (~ 100 kJ mol^{-1}) processes are responsible for the cardiac glycoside's relatively slow kinetics of

association and dissociation. The high value of ΔG_f^* and the high affinity suggest that the cardiac glycoside has restricted access to a specific binding site.

Fig. 6a and b also show that the entropy and enthalpy changes for the overall association are similar for ouabain and AO. In both cases, the overall association is driven by substantial contributions from both enthalpy and entropy. These similarities suggest that once AO is bound to the enzyme, the interactions which are responsible for the highly specific binding of ouabain are for the most part unaffected by the anthracene moiety.

However, there are significant interesting differences for the transition state. AO has a much larger activation enthalpy than ouabain. In addition, the transition state for AO is entropically favored, unlike for ouabain where the entropy of the transition state is intermediate between the unbound and bound state entropies. These substantial differences in the transition state entropies and enthalpies suggest that the binding process is different for ouabain and AO. It may be that for AO, the additional size due to the anthracene moiety requires that more noncovalent bonds be broken as the complex passes through the transition state. Breaking these additional bonds would require a larger increase in enthalpy than is required by ouabain. Further, if more bonds are broken in the transition state with AO, this could result in increased entropy, which agrees with what we observe for the transition state of the AO-enzyme complex. Thus, the transition state enthalpies and entropies are consistent with the existence of a restricted access binding pocket. Further support comes from the enhanced fluorescence intensity and spectral shifts of AO bound to the enzyme which suggest that the anthracene is in a hydrophobic environment protected from water [4].

It is interesting that the entropically favored nature of the binding of the cardiac glycosides ouabain and AO to the Na/K-ATPase is shared by other inhibitor binding including anesthetic binding to membrane bound Ca-ATPase [20] and antagonist binding to the β-adrenergic receptor [21]. In each of these, the inhibitor is binding to a membrane bound protein, although the nature of the binding site is different in each case. The anesthetic binds with relatively low affinity to hydrophobic pockets. The antagonists and cardiac glycosides bind with higher affinities, but while the antagonists block ligand from a specific binding site, the cardiac glycosides do not compete for substrate or ligand binding sites. The cardiac glycosides apparently have their own specific binding site, which may be a regulatory site for endogenous ouabain-like-factors, a subject which has been and continues to be an area of great interest [22–27].

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Appendix A

The exact solution for the association of AO with enzyme depicted in Eq. (1) is found by solving the differential equation

$$\frac{1}{k_{on}}\frac{\partial C}{\partial t} = E_t AO_t - (E_t + AO_t + K_d)C + C^2 \qquad (8)$$

where C is the concentration of AO-enzyme complex and E_t and AO_t are the total concentrations of enzyme and AO, respectively. The initial condition is that there is no complex, $C(t=0)=0$. The solution for the fluorescence signal is then

$$F(t) = F_0 + \Delta F \frac{1 - \exp\left(-\sqrt{b^2 - 4d}\; k_{on} t\right)}{1 - \frac{a_2}{a_1}\exp\left(-\sqrt{b^2 - 4d}\; k_{on} t\right)} \qquad (9)$$

where

$$a_{1.2} = \frac{1}{2}\left(b \pm \sqrt{b^2 - 4d}\right) \qquad (10)$$

and $b = (E_t + AO_t + K_d)$ and $d = E_t AO_t$. Eq. (9) is valid except in the special case $E_t = AO_t$ and $K_d = 0$ (irreversible binding). For completeness, we include the solution of Eq. (8) for this special case.

$$F(t) = F_0 + \Delta F\left(\frac{E_t k_{on} t}{1 + E_t k_{on} t}\right) \qquad (11)$$

If $AO_t \gg E_t$, then the value of k_{on} retrieved from the approximate solution, Eqs. (2) and (3), agrees with the true value obtained from the exact solution, Eq. (9). As AO_t is reduced towards E_t, the value of k_{on} from the approximate solution deviates below the true value. However, using values for E_t, AO_t, and K_d corresponding to our experimental conditions, the deviation reaches a maximum of 6% near $AO_t = 4E_t$. Further reduction to $AO_t = E_t$ causes k_{on} from the approximate solution to turn around and increase above the true value. The result is that a plot of the observed rate versus AO_t is nearly linear not only for values of $AO_t \gg E_t$ but also for values of AO_t approaching E_t. We calculate that for the association experiments plotted in Fig. 2, AO_t ranges from 4 to 30 times E_t, where E_t was determined by measured Na/K-ATPase activities [28] and confirmed by calculating E_t from the best fit value of the ratio of a_2/a_1 in Eq. (9) when fit to the lowest AO concentration association experiment. Over this range of AO, the slope of the line according to Eq. (3) gives a value for k_{on} within 2% of the true value.

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