Stoichiometry and Dissociation Constants for Interaction of Tetracaine with Mitochondrial Adenosinetriphosphatase As Determined by Fluorescence

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ABSTRACT: The stoichiometry and dissociation constants for the interaction of tetracaine with chloroform-released ATPase prepared from beef heart mitochondria were determined from the enhancement of tetracaine fluorescence intensity that occurs upon binding. There is a single class of approximately 60 thermodynamically equivalent binding sites on ATPase for tetracaine; these have a microscopic dissociation constant of 4.9×10^{-4} M at 25 °C under solvent conditions that are similar to those used for enzyme assay. Analysis of enzyme kinetic data according to a partial noncompetitive scheme gave an inhibitor constant for tetracaine of 4.8×10^{-4} M. The numerical agreement between the dissociation constant and the inhibitor constant shows that the filling of the same class of sites is probably responsible for both the enzyme inhibition and the fluorescence enhancement. The sites are hydrophobic, as evidenced by the blue shift and the magnitude of the fluorescence enhancement that occur upon binding.

The objective of our work is to find out how local anesthetics and related compounds cause inhibition of mitochondrial ATPase. Compounds such as tetracaine, dibucaine, lidocaine, benzocaine, and tricyclic antipsychotics and antidepressants (e.g., chlorpromazine) cause the partial, reversible inhibition of ATPase activity. This occurs in submitochondrial particles (Chazotte et al., 1982), F_1ATPase, and chloroform-released ATPase (Penefsky et al., 1960; Palatini, 1982; Vanderkooi et al., 1981; Chazotte et al., 1982; Adade et al., 1984). One or more of these compounds has also been shown to inhibit the homologous ATPases isolated from other sources, including TF_1 from the thermophilic bacterium PS3 (Saishu et al., 1983; Bullough et al., 1985), *Mycobacterium phlei* ATPase (Agarwal & Kalra, 1984), *Escherichia coli* ATPase (Bullough et al., 1985), plant mitochondria ATPase (Dunn et al., 1984), and chromaffin granule ATPase (Weinbach et al., 1983). The details of the inhibitory characteristics and the degree of sensitivity to particular agents differ according to the source of the ATPase.

Several lines of evidence indicate that, in addition to enzyme inhibition, the local anesthetics cause conformational alterations of mitochondrial ATPase on the tertiary or quaternary level. Chazotte et al. (1982) found that tetracaine changed the ultracentrifugal schlieren pattern of ATPase in a manner indicative of a reversible dissociation of the subunits. Adade (1985) showed that the SH group reactivity of ATPase is increased in the presence of local anesthetics. The differential scanning calorimetry results of Kresheck et al. (1985) showed that anesthetics facilitate thermal denaturation, as evidenced by the shift to progressively lower temperatures of the denaturational transition as a function of anesthetic concentration (e.g., from 80.5 °C in the absence of anesthetic to 70 °C with 1 mM tetracaine or 72 °C with 0.3 mM dibucaine). By contrast, these same concentrations of anesthetics protect the enzyme from cold lability (Vanderkooi et al., 1985; Adade, 1985). Chlorpromazine and other tricyclics also protect against cold lability, as has been shown by Palatini (1982) and Bullough et al. (1985).

The lowering of the thermal denaturational transition and the protection against cold lability are evidence that the anesthetics affect the inter- or intrasubunit forces in a highly temperature-dependent manner. Cold lability has been shown to be the result of subunit dissociation, and chaotropic salts such as KNO_3 accelerate the dissociation (Penefsky & Warner, 1965). The low temperature effects of anesthetics are therefore opposite to those of the chaotropic salts but similar to the effects of alcohols, which have also been reported to confer cold stability (Penefsky & Warner, 1965).

The objective of this study was to determine how many anesthetic molecules are bound to chloroform-released ATPase at enzyme inhibitory concentrations. Analysis of enzyme kinetic data yielded the inhibitor constant K_i, and fluorescence measurements were used to determine the dissociation constant (K) and number of binding sites. K and K_i were then compared and were found to be equal to within experimental error; this showed that the enzyme–tetracaine interactions that gave rise to the fluorescence changes are probably the same interactions that cause enzyme inhibition. The fluorescence data indicate that there are many (on the order of 60) binding sites for tetracaine on ATPase, all with similar dissociation constants.

Tetracaine has a strong absorption band centered at 310 nm and fluoresces with a maximum intensity between 350 and 370 nm, depending upon the solvent. Elferink (1977) reported that the tetracaine fluorescence intensity increased 20-fold upon transfer from a neutral aqueous buffer to 95% ethanol, with a concomitant blue shift. He also showed that the intensity increased upon binding of tetracaine to erythrocyte membranes. Garcia-Soto and Fernandez (1983) showed that a change from pH 7 to pH 12 caused a 2-fold increase in the tetracaine fluorescence intensity, with a negligible blue shift, but addition of Triton X-100 to the alkaline solution gave a 25-fold enhancement of intensity. These observations show that a strong enhancement of fluorescence intensity coupled with a blue shift is evidence for the transfer of tetracaine to a less polar environment.

Materials and Methods

Enzyme Preparation and Assay. Chloroform-released ATPase was prepared from beef heart submitochondrial particles as previously described (Kresheck et al., 1985; Beechey et al., 1975; Linnett et al., 1979). This material has

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a maximal activity of about 14 μmol of ATP hydrolyzed min^{-1} (mg of protein)$^{-1}$. Lowe and Beechey (1982) have investigated the reasons for the lower specific activity of this type of preparation as compared to the F_1ATPase prepared by other methods, which give rates of 60–140 μmol min^{-1} (mg of protein)$^{-1}$. They determined that the chloroform-released ATPase is "at least 90% pure", as judged by polyacrylamide gel electrophoresis in the presence and absence of sodium dodecyl sulfate. [Fisher et al. (1981) also claimed that beef heart chloroform-released ATPase is "nearly homogeneous", and our own polyacrylamide gel electrophoresis studies are in agreement with these reports.] Hence, the low activity of the chloroform-released ATPase is not due to contamination by other proteins. Lowe and Beechey (1982) concluded that F_1ATPase and chloroform-released ATPase are allomorphs; that is, they are forms of the same enzyme that differ in certain physical and enzymatic properties. They further point out that the properties of the chloroform-released ATPase are in some ways more similar to those of the membrane-bound ATP synthetase than are those of F_1ATPase and suggest that the chloroform-released enzyme may in fact provide a better model system for the synthetase.

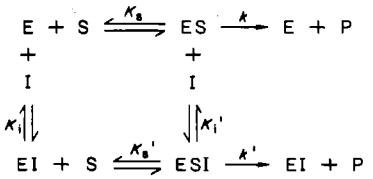
ATPase activity was determined with the ATP regenerating system of Pullman et al. (1960), as described by Adade et al. (1984). Protein concentration was determined according to Lowry et al. (1951) with bovine serum albumin as the standard.

Buffers. ATPase was prepared in "sucrose buffer", which consisted of 0.25 M sucrose, 10 mM tris(hydroxymethyl)-aminomethane sulfate (Tris sulfate), and 1 mM ethylenediaminetetraacetic acid (EDTA), pH 7.5. "Sucrose–Tris buffer" contained 9 parts of sucrose buffer plus 1 part of 500 mM Tris acetate, pH 7.5; this gave a final Tris acetate concentration of 50 mM, which is the same as its concentration in the ATPase assay medium. "Phosphate buffer" was 0.05 M potassium phosphate, pH 7.0.

All biochemical reagents were obtained from Sigma. Tetracaine hydrochloride [4-(butylamino)benzoic acid 2-(dimethylamino)ethyl ester hydrochloride] was used as purchased without further purification.

Spectral Measurements. Absorption spectra were recorded with a Hitachi Model 110A double-beam recording spectrophotometer. Fluorescence spectra were measured as previously described (Vanderkooi, 1984), with an SLM-Aminco Model 4800S spectrofluorometer. Quantum yields were determined by using quinine as the standard, with the quantum yield of quinine being taken as 0.70 (Scott et al., 1970). All measurements were made at 25 °C.

Kinetic Analysis for Partial Noncompetitive Inhibition. We have shown previously that the inhibition of ATPase by local anesthetics is partial and noncompetitive (Adade et al., 1984). This type of kinetics may be analyzed according to the following two-state model, which assumes rapid equilibrium among the enzyme species (Segal, 1976):



(1)

The inhibitor, I, can bind to both the E and ES states of the enzyme with dissociation constants of K_i and K_i', respectively, which may or may not be equal. (The constraint applies that $K_sK_i' = K_iK_s'$). For partial inhibition, ES and ESI can both yield products, but with $k' < k$. The reaction rate v is proportional to the amounts of ES and ESI present:

$$v = k[ES] + k'[ESI] \tag{2}$$

The parameters in eq 1 may be determined from a set of data of rate vs. substrate concentration at several inhibitor concentrations. Under conditions of saturating substrate ($1/[S] = 0$ on reciprocal plots), all enzyme is present as ES or ESI. One may then determine k from the rate in the absence of inhibitor using the relationship $k = V_{max}/[E_t]$. In the presence of inhibitor, the fraction of the enzyme that is in the ESI state is given in terms of K_i' as β:

$$\beta = \frac{[I]/K_i'}{1 + [I]/K_i'} \tag{3}$$

Equation 2 may be also written in terms of β:

$$v/[E_t] = (1 - \beta)k + \beta k' \tag{4}$$

Substituting V_{max}/k for $[E_t]$ gives

$$(V_{max} - v)/V_{max} = \beta(k - k')/k \tag{5}$$

Combining eq 3 and 5 yields

$$V_{max}/(V_{max} - v) = \frac{kK_i'}{k - k'}\frac{1}{[I]} + \frac{k}{k - k'} \tag{6}$$

k' and K_i' may then be determined from the slope and intercept of a plot of $V_{max}/(V_{max} - v)$ vs. $1/[I]$, since k is already known.

The general reciprocal form of the equation relating v and [S] as a function of I for the scheme given in eq 1 is as follows:

$$\frac{1}{v} = \frac{K_s\left(1 + \dfrac{[I]}{K_i}\right)}{V_{max}\left(1 + \dfrac{k'[I]}{kK_i'}\right)}\frac{1}{[S]} + \frac{1 + \dfrac{[I]}{K_i'}}{V_{max}\left(1 + \dfrac{k'[I]}{kK_i'}\right)} \tag{7}$$

The coefficient of $1/[S]$ gives the slopes, and the second term equals the intercepts of $1/v$ vs. $1/[S]$ plots. For partial noncompetitive inhibition, replots of the slopes and intercepts as a function of [I] will give convex hyperbolic curves. The slope to intercept ratio is given by eq 8. If this ratio is constant

$$\frac{\text{slope}}{\text{intercept}} = \frac{K_s(1 + [I]/K_i)}{1 + [I]/K_i'} \tag{8}$$

as a function of [I], then the parenthesized term in the numerator and the denomenator are equal, so that $K_i = K_i'$ and slope/intercept = K_s.

This kinetic analysis was required since the objective was to determine K_i for comparison with the dissociation constant K determined by fluorescence. K_i, rather than K_i', must be used in this comparison since the fluorescence measurements were made in the absence of substrate. It turns out, however, that K_i and K_i' are equal, confirming that the inhibition is of a pure noncompetitive type.

Ratiometric Method for Determining Dissociation Constants from Fluorescence Data. The methods that have previously been described for studying the association of compounds such as 8-anilinonaphthalene-1-sulfonate (ANS) with macromolecules use the assumption that the fluorescence of unbound ligand is negligible in comparison with the fluorescence of the bound molecules [e.g., Cantley and Hammes (1976) and Torgerson et al. (1979)]. This method cannot be used for tetracaine binding to ATPase since the fluorescence intensities of the bound and free molecules are of a similar order of magnitude. We have instead used the non-zero fluorescence of the unbound ligand molecules to advantage and have developed a ratiometric method for studying the

tetracaine–protein association. In this method, the fluorescence intensity is measured for a pair of matched tetracaine solutions, one with and the other without protein. The absorbancies of the two solutions are initially made equal at both the excitation and emission wavelengths by the addition, if necessary, of appropriate nonfluorescent chromophores to the protein-free solution. Both solutions are then titrated in an identical manner so as to maintain equal absorbancies throughout the course of an experiment. By this means, the primary and secondary inner filter effects cancel when the ratio of fluorescence intensities is taken, and the need to use empirical correction formulas for inner filter effects is avoided.

The equations for the ratiometric method were derived as follows. Let f_f and f_b equal the molar fluorescence increments for free and bound ligand, respectively. F_{obsd} is the measured fluorescence in the presence of protein, and F_0 is the fluorescence of the matched protein-free solution. [L] is the total ligand concentration, and α is the fraction of bound ligand. This gives

$$F_{obsd} = [(1 - \alpha)f_f + \alpha f_b][L] \quad (9)$$

$$F_0 = f_f[L] \quad (10)$$

Taking the ratio of eq 9 and 10 gives

$$(F_{obsd}/F_0 - 1) = \alpha(f_b/f_f - 1) \quad (11)$$

Setting $F_{obsd}/F_0 - 1$ equal to R and $f_f/f_b - 1$ to r, we get $\alpha = R/r$.

The number of moles of ligand bound per mole of protein, ν, is given by eq 12 for the case of a single class of identical

$$\nu = \frac{n[L_f]/K}{1 + [L_f]/K} \quad (12)$$

independent sites; n is the total number of sites and K is the microscopic dissociation constant (Cantor & Schimmel, 1980). $[L_f]$ is the concentration of unbound ligand. ν may also be expressed in terms of the total ligand concentration as

$$\nu = \alpha[L]/[P] \quad (13)$$

where [P] is the protein concentration. Manipulation of eq 12 and 13 yields the following equation for α:

$$\frac{1}{\alpha} = 1 + \frac{K}{n - \nu}\frac{1}{[P]} \quad (14)$$

In the limit that $1/[P]$ goes to zero, ν also approaches zero and can be dropped from the equation. Substituting R/r for α gives

$$\frac{1}{R} = \frac{1}{r} + \frac{K}{rn}\frac{1}{[P]} \quad (15)$$

The slope of a plot of $1/R$ vs. $1/[P]$ at constant [L] will equal $K/(rn)$, and the intercept at $1/[P] = 0$ will give $1/r$.

Once the value of r is known, n may be determined in a separate experiment in which the protein concentration is held constant but the ligand concentration is varied; ν is related to the ligand concentration as

$$\frac{1}{\nu} = \frac{1}{n} + \frac{K}{n}\frac{1}{[L_f]} \quad (16)$$

ν is calculated from eq 13 with α being determined experimentally as R/r, and $[L_f] = (1 - \alpha)[L]$. Plotting $1/\nu$ vs. $1/[L_f]$ gives $1/n$ as the intercept and K/n as the slope, in the limit of large [L].

In the limit that [L] goes to zero, the following limiting relationship is obtained from eq 12:

$$\nu \simeq (n/K)[L_f] \quad (17)$$

Thus for small [L], the slope of a plot of ν vs. $[L_f]$ gives n/K; this value will pertain to the strongest binding sites, if all sites are not equivalent. If the value of n/K obtained by plotting the low concentration data according to eq 17 is the same as that obtained from the high concentration data with eq 16, all sites evidently have similar dissociation constants.

Experimental Setup. The ratiometric fluorescence measurements were made at excitation and emission wavelengths of 320 and 350 nm, respectively. These values were chosen to maximize the difference in intensity of the bound and free ligand molecules. Under these conditions, Raman scattering and the fluorescence of the tetracaine-free protein and buffer solutions were also very weak. The absorbances of the protein-free buffer solutions were adjusted to equal those of the protein solution at 320 and 350 nm by the addition of low concentrations of chlorpromazine (which absorbs more strongly at 320 nm) and ANS (stronger absorbance at 350 nm). The protein solution absorbancies at the two wavelengths in question were never greater than 0.05; hence, the ANS and chlorpromazine concentrations required to match these values were less than 1×10^{-5} M. Control experiments confirmed that these substances did not contribute to the fluorescence of the buffer solutions.

A set of four solutions was used in experiments in which the protein concentration was varied. Solution I contained ATPase + tetracaine; II contained buffer + tetracaine; III contained ATPase at the same concentration as in I; IV contained buffer. Solutions I and II were stepwise diluted with a solution containing the same tetracaine concentration as initially present, and solutions III and IV were diluted with the same volumes of buffer. The fluorescence intensities (F_I–F_{IV}) of the four solutions were measured after each addition. The value of R was calculated as

$$R = \frac{F_I - F_{III}}{F_{II} - F_{IV}} - 1 \quad (18)$$

For titrations with tetracaine at constant protein concentration, two solutions were used: Solution I contained protein, and II was protein-free. The titrant for solution I contained protein (at the same concentration as in I) and tetracaine, and the titrant for II contained only tetracaine. R was calculated according to eq 18 by setting F_{III} and F_{IV} equal to the fluorescences of solutions I and II, respectively, before the addition of tetracaine. Gilmont microburets were used in all experiments for the delivery of titrant.

The ATPase concentration was calculated as in our earlier work (Kresheck et al., 1985), assuming a M_r of 360000 and that all protein present is ATPase. The errors that will result from the extent to which these assumptions are incorrect will be of a systematic nature and should not invalidate the principal conclusions arrived at in this work.

RESULTS

Spectral Properties of Tetracaine. The UV absorption spectrum of tetracaine has a single symmetrical peak between 250 and 350 nm; in neutral aqueous media, the maximum is at 310 nm, but it shifts to 302 nm at pH 11. In absolute ethanol the maximum is at 308 nm. In pH 7.0 phosphate buffer, the extinction coefficient at 310 nm is 23.4 mM^{-1} cm^{-1}, whereas in absolute ethanol it is 29.0 mM^{-1} cm^{-1} at 308 nm. Figure 1 shows the spectra in ethanol and in phosphate buffer. (The spectrum in sucrose buffer is nearly identical with that in phosphate buffer.)

The fluorescence emission of tetracaine occurs between 320 and 450 nm. The emission maximum is at 368 nm in neutral aqueous buffers and shifts only slightly, to 365 nm, at pH 11.

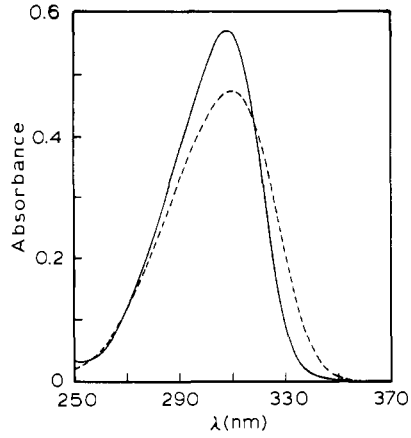


FIGURE 1: Absorption spectra of tetracaine: (solid line) 2×10^{-5} M tetracaine hydrochloride in absolute ethanol; (dashed line) 2×10^{-5} M tetracaine hydrochloride in 0.05 M potassium phosphate buffer, pH 7.0.

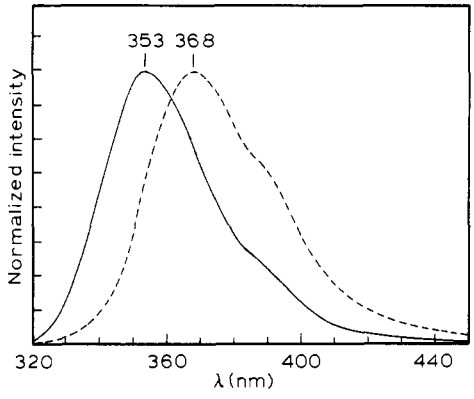


FIGURE 2: Fluorescence emission spectra of tetracaine, corrected and normalized to equal peak height. The solvent spectra were digitally subtracted before plotting. (Solid line) 4×10^{-7} M tetracaine hydrochloride in absolute ethanol; (dashed line) 2×10^{-5} M tetracaine hydrochloride in 0.05 M potassium phosphate buffer, pH 7.0. Excitation was at 310 nm with a 4-nm band-pass. The emission band-pass was 2 nm.

A larger blue shift is seen in absolute ethanol, to 353 nm. The fluorescence intensity and quantum yield are about twice as great at pH 11 as at pH 7, but a much larger increase occurs in ethanol. At pH 7.0 in phosphate buffer the quantum yield is 0.0073, but in absolute ethanol it is 0.35. In sucrose buffer, the emission spectrum and quantum yield are essentially the same as in phosphate buffer. The normalized, corrected emission spectra for tetracaine in phosphate buffer and in ethanol are shown in Figure 2; Figure 3 includes an uncorrected spectrum in sucrose buffer.

The tetracaine emission spectrum is blue-shifted in the presence of ATPase and is of greater intensity than in protein-free buffer at the same concentration. This is shown in Figure 3. These spectral effects of ATPase are similar to those that occur upon transfer of tetracaine from water to ethanol and are also similar to the spectral changes in the presence of membranes (Elferink, 1977) or Triton X-100 (Garcia-Soto & Fernandez, 1983). The molecules of tetracaine bound to ATPase and therefore evidently in a relatively nonpolar environment. All tetracaine concentrations employed in these studies were far below the critical micellar concentration of 70 mM reported by Fernandez & Calderon (1980).

Determination of Dissociation Constants from Fluorescence Data. The enhancement of tetracaine fluorescence in the presence of ATPase provided the means of determining the dissociation constants and the number of binding sites. Two

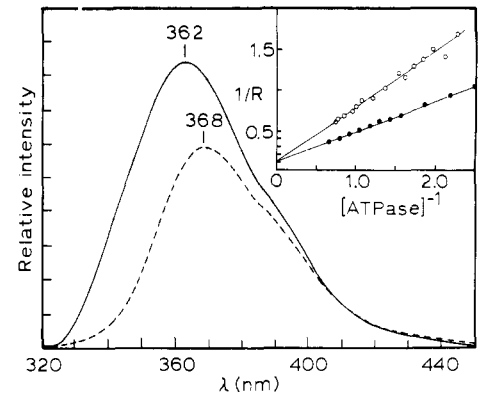


FIGURE 3: Relative fluorescence intensity (uncorrected) of tetracaine in the presence and absence of ATPase. The tetracaine concentration was 1.05×10^{-5} M in each case. (Solid line) Tetracaine in the presence of 0.65 mg/mL ATPase, in sucrose buffer; (dashed line) tetracaine in sucrose buffer. The spectra of protein and buffer solutions without tetracaine were digitally subtracted in each case before plotting. The instrumental settings were the same as are given in the legend to Figure 2. (Inset) A representative plot showing the dependence of the relative fluorescence intensity on the reciprocal of the protein concentration. $1/R$ is as defined in the text. The units on the ATPase concentration axis are μM^{-1}. The filled circles are for ATPase in sucrose buffer and the open circles for sucrose–Tris buffer. The initial protein concentration was 0.56 and 0.60 mg/mL for sucrose buffer and sucrose–Tris buffer, respectively. The tetracaine concentration was 3.1 μM in the sucrose buffer experiment and 5.4 μM in sucrose–Tris.

types of experiments were carried out. In the first, the tetracaine concentration was held constant while the protein was diluted, giving data at a range of protein concentrations; and in the second, the protein concentration was unchanged while the tetracaine concentration was increased from an initially zero value. Both types of experiments were carried out in sucrose buffer and in sucrose–Tris buffer. The protein dilution experiments were analyzed according to eq 15 and the tetracaine titration experiments according to eq 16 and 17. The ratio of fluorescence increments of bound and free tetracaine was determined from the protein dilution experiments; this value was needed in order to interpret the tetracaine titration experiments, which in turn yielded n, the number of binding sites. Both types of experiments gave estimates of K/n.

The results of the protein dilution experiments are shown in the inset of Figure 3 for ATPase in sucrose and sucrose–Tris buffers. The intercepts are the same in both buffers, giving a value for $1/r$ of 0.102 ± 0.007, averaged over four determinations. From this, the ratio of fluorescence increments (f_b/f_f) was calculated to be 10.8 ± 0.7. The slopes, which equal $K/(rn)$, differ markedly between the two buffers, showing that K/n is sensitive to the buffer composition. Table I includes the values of K/n computed from these slopes.

The value of n was determined from titration experiments in which the protein concentration was held constant at 0.6 mg/mL and the tetracaine concentration was varied over the range of 0.23–70 μM. (Solutions of tetracaine of the latter concentration had an absorbance of about 1.6 at the excitation wavelength and constituted the practical upper limit of concentration at which reliable ratiometric fluorescence data could be obtained.) Plots of $1/\nu$ vs. $1/[\text{tetracaine}]_{\text{free}}$ were linear for the titrations in both buffer systems (sucrose and sucrose–Tris), which implies that there is only a single class of binding sites. The intercepts of these plots, which equal $1/n$, were the same for both buffer systems, having a value of 0.017 ± 0.012, averaged over three determinations. This yields a mean value for n of 60, but with a range of 35–200. The slopes of the plots, which equal K/n, differed for the two buffer systems in the same manner as in the protein dilution ex-

Table I: Tetracaine–ATPase Dissociation Constants Determined by Fluorescence[a]

method	sucrose buffer		sucrose–Tris buffer	
	K/n ($\times 10^{-6}$ M)	K ($\times 10^{-4}$ M)[b]	K/n ($\times 10^{-6}$ M)	K ($\times 10^{-4}$ M)[b]
protein dilution[c]	3.82 ± 0.1	2.29 ± 0.04	8.2 ± 1.5	4.6 ± 0.9
ν vs. [tetracaine][d]	4.2	2.5	8.4 ± 0.5	5.0 ± 0.3
$1/\nu$ vs. $1/$[tetracaine][e]	4.1	2.4	8.0 ± 0.7	4.8 ± 0.4
mean value ± SD	4.0 ± 0.2	2.4 ± 0.1	8.2 ± 0.2	4.9 ± 0.1

[a] The values shown are based on the average of two experiments in cases where a range is given and on a single experiment in the remaining entries. [b] The value of K was calculated from K/n using $n = 60$. [c] K/n was calculated from the slope of the plot of $1/R$ vs. $1/$[ATPase], using $1/r = 0.102$. [d] Calculated according to eq 17. [e] Calculated according to eq 16.

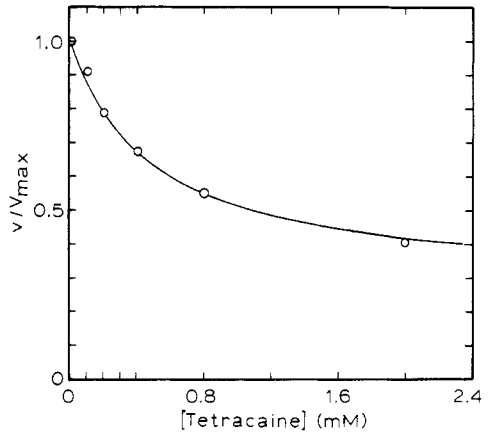


FIGURE 4: Comparison of the experimental and computed dependence of relative ATPase activity on tetracaine concentration, at saturating ATP concentration. The circles are the experimental points, and the solid line was calculated from the equations and parameters given in the text.

periments. K is therefore buffer-dependent, with K being larger in sucrose–Tris than in sucrose buffer. The experimental results are summarized in Table I.

The titration data for low tetracaine concentrations (0.23–5 μm) were plotted as ν vs. $[\text{tetracaine}]_{\text{free}}$ according to eq 17. The slopes, which equal n/K, are included in Table I, expressed for consistency as K/n. The K/n values obtained in this manner for the low concentration data agree with the values obtained from reciprocal plots for higher concentrations (5–70 μM). This agreement confirms that there is only a single class of equivalent binding sites.

Determination of Tetracaine Inhibitor Constant from Kinetic Data. The ATPase inhibition data of Adade et al. (1984) were analyzed according to eq 1 with the procedures described under Materials and Methods. The value of k was determined to be 10.3 μM min^{-1} mg^{-1} for this particular preparation. The value of k' was then determined by plotting the intercept values as $V_{max}/(V_{max} - v)$ vs. $1/$[tetracaine], according to eq 6. k' and K_i' were calculated to be 2.8 μM min^{-1} mg^{-1} and 4.8 $\times$ 10^{-4} M, respectively. The ratio of k'/k was therefore 0.275. The value of K_s was determined to be 0.95 $\times$ 10^{-4} M. Finally, it was concluded that $K_i = K_i'$, hence also $K_s' = K_s$, since the slope to intercept ratio (eq 8) was constant as a function of tetracaine concentration. The equivalence of K_i and K_i' confirms that the inhibition is noncompetitive.

The validity of the parameters thus derived was tested by comparing the experimental rates at saturating substrate concentration with the calculated degree of inhibition. This result is plotted in Figure 4. The theoretical slope and intercept replots were also calculated and are compared with the experimental values in Figure 5. The agreement between the experimental and computed values is good. [Adade (1985) has analyzed the inhibition data by computer using a weighted nonlinear least-squares method; the numerical results obtained were the same as derived here by essentially graphical methods.]

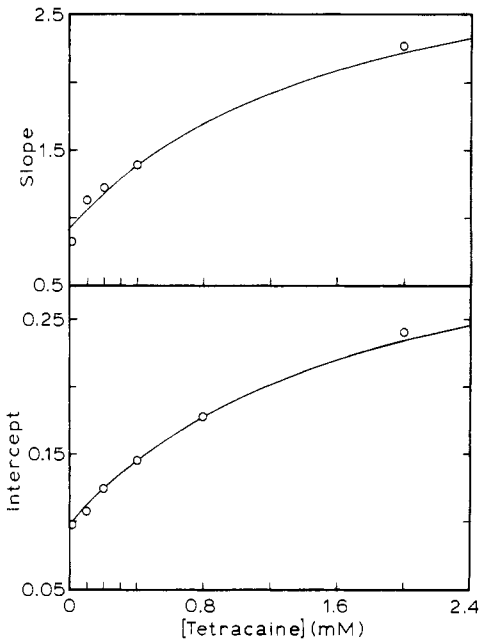


FIGURE 5: Calculated and experimental slope and intercept replots for ATPase inhibition as a function of the tetracaine concentration. The units for the intercept values are $(\mu\text{M min}^{-1}\text{ mg}^{-1})^{-1}$, and the units for the slopes are $(\mu\text{M min}^{-1}\text{ mg}^{-1})^{-1}$ M $\times$ 10^5. The circles are the experimental points, and the solid lines were calculated from the equations and parameters given in the text.

In light of the fluorescence data that indicate that there are many binding sites for tetracaine, we asked whether there is any kinetic evidence for cooperative inhibition resulting from multiple site binding. Hill plots were therefore prepared by plotting ln $[\beta/(1-\beta)]$ vs. ln [tetracaine], for various substrate concentrations (Cantor & Schimmel, 1980). The slope of these plots, which is the Hill constant, was equal to 1 in all cases, showing that there is no cooperativity among inhibitor molecules.

Discussion

The principal result of the present work is that there are on the order of 60 thermodynamically equivalent binding sites for tetracaine on ATPase which have a microscopic dissociation constant of 0.49 mM in sucrose–Tris buffer. This value was found to be equivalent to the enzyme inhibitor constant (0.48 mM.) The kinetic measurements were made in a buffer of similar composition to sucrose–Tris buffer, and hence, the value of K determined in this buffer is used for comparison with K_i. No evidence was found for a small class of tight binding sites; the existence of such should have been revealed by analysis of the fluorescence data at low concentration with eq 17.

Since all of the tetracaine binding sites have the same dissociation constant, which in turn equals the inhibitor constant determined according to the model given in eq 1, it means

that the fraction of binding sites filled is directly proportional to the degree of partial enzyme inhibition. The inhibition scheme in eq 1 is based on a two-state model, however, in which it was assumed that the binding of only a single inhibitor molecule causes the transition from the state of higher activity to that of lower activity. Does this mean that of the roughly 60 binding sites identified by fluorescence only one, which is thermodynamically indistinguishable from all the rest, is responsible for the inhibition? We will show that an alternative model, based on the assumption of linearly additive cumulative inhibition, yields the same rate equations as obtained from eq 1 and hence can equally well account for the data in hand.

From the fluorescence data, we know we are dealing with a multiple equilibrium problem involving the binding of ligand to identical, independent sites. This gives a total of $n + 1$ enzyme species, all of which are acitve but with possibly different rate constants. The observed rate will be the sum of the rates from the individual components, defined as

$$\begin{aligned} \mathrm{ES} &\xrightarrow{k_0 = k} \mathrm{E} + \mathrm{P} \\ \mathrm{ESI} &\xrightarrow{k_1} \mathrm{EI} + \mathrm{P} \\ \mathrm{ESI_2} &\xrightarrow{k_2} \mathrm{EI_2} + \mathrm{P} \\ &\vdots \\ \mathrm{ESI}_n &\xrightarrow{k_n = k'} \mathrm{EI}_n + \mathrm{P} \end{aligned} \tag{19}$$

At saturating inhibitor concentration all sites are filled, and thus, $k_n = k'$, whereas in the absence of inhibitor no sites are filled so that $k_0 = k$. The observed rate will be given by eq 20. The condition of linearly additive cumulative inhibition

$$v = \sum_{i=0}^{n} k_i[\mathrm{ESI}_i] \tag{20}$$

implies that the k_i values change in a linear manner from k to k' in proportion to the value of i. This means that

$$k_i = k - \frac{i}{n}(k - k') \tag{21}$$

Substitution into eq 19 yields

$$v = \sum_{i=0}^{n}\left[k - \frac{i}{n}(k - k')\right][\mathrm{ESI}_i] \tag{22}$$

$$v = k\sum_{i=0}^{n}[\mathrm{ESI}_i] - \frac{k - k'}{n}\sum_{i=0}^{n} i[\mathrm{ESI}_i] \tag{23}$$

Under conditions of saturating substrate, $\sum_{i=0}^{n}[\mathrm{ESI}_i] = [\mathrm{E_t}]$, the total enzyme concentration; hence

$$\frac{v}{[\mathrm{E_t}]} = k - \frac{k - k'}{n}\frac{\sum_{i=0}^{n} i[\mathrm{ESI}_i]}{[\mathrm{E_t}]} \tag{24}$$

The ratio $\sum_{i=0}^{n} i[\mathrm{ESI}_i]/[\mathrm{E_t}]$ equals ν, the average number of moles of ligand bound per macromolecule. This expression has been evaluated by Cantor and Schimmel (1980) in terms of the ligand dissociation constant, K, for the applicable case of identical independent sites, with the following expression being obtained:

$$\sum_{i=0}^{n}\frac{i[\mathrm{ESI}_i]}{[\mathrm{E_t}]} = \frac{n[\mathrm{I}]/K}{1 + [\mathrm{I}]/K} \tag{25}$$

Comparison of eq 25 with eq 3 shows that the right side equals $n\beta$, since it was shown empirically that the inhibitor constant K_i' (which also equals K_i) is equal to the dissociation constant K. When this result is substituted back into eq 24, the n's cancel and the final equation is identical with that given in eq 4 for the two-state model:

$$\frac{v}{[\mathrm{E_t}]} = k - (k - k')\beta \tag{26}$$

We see therefore that it is impossible to determine from the kinetic data the number of sites that contribute to the inhibition, since n does not appear in the final equation. The cumulative inhibition model used here does not entail the assumption that all molecularly defined binding sites individually contribute equally to the observed inhibition, but rather that the degree of inhibition is proportional to the mean number of sites that are filled. Since all sites have the same dissociation constant, they will be filled in a random manner, and the k_i will be the rate constants averaged over all ways of filling i of the n sites.

In molecular terms, there are many examples of enzyme inhibition being caused by the binding of a single inhibitor molecule, but it may not be immediately obvious how there could be cumulative inhibition resulting from the filling of many sites. The blue shift and increase in tetracaine fluorescence intensity show that the binding sites are hydrophobic; this conclusion was also arrived at by Chazotte et al. (1982), who showed that the inhibitory potencies of the various local anesthetics on ATPase are proportional to their octanol/water partition coefficients. We may therefore suggest that the inhibitor molecules lodge in hydrophobic clefts or pockets between the ATPase subunits, thereby causing a change in the relative positioning of the subunits. It is quite plausible to imagine that a geometrical alteration of this type could occur in a stepwise, additive manner, the magnitude of the change and the degree of inhibition thereby caused being proportional to the number of anesthetic molecules that are bound. This picture could also explain the lowering of the denaturational phase transition temperature, as observed by Kresheck et al. (1985), which occurs over the same range of tetracaine concentrations that give enzyme inhibition.

The fact that enzyme inhibition is partial rather than complete, with considerable residual activity remaining at high anesthetic concentration, shows that the structural perturbation caused by tetracaine is not of a drastic nature. Dibucaine and chlorpromazine are also partial inhibitors of ATPase (Chazotte et al., 1982), but both of these cause a greater maximal degree of inhibition than tetracaine [see Figure 1 of Chazotte et al. (1982)]. These differences in inhibitory effectiveness can be explained in terms of the present hypothesis by supposing that the magnitude of the geometrical change caused by the intercalation of these di- and tricyclic compounds into hydrophobic pockets is greater than that for the insertion of the monocyclic tetracaine molecule. (The fact that the various anesthetics give different levels of residual activity with the same enzyme preparation is also strong evidence against the possibility that the observed partial inhibition is due to two populations of ATPase, one being sensitive and the other insensitive to inhibition by anesthetics. If such were the case, the same maximal degree of inhibition should be given by all of the anesthetics.)

It was pointed out in the introduction that the same concentrations of tetracaine and other local anesthetics that cause enzyme inhibition also protect ATPase from cold denaturation (Vanderkooi et al., 1985; Adade, 1985). This observation may be understood if one assumes that the intercalation of anesthetic molecules between the ATPase subunits causes a strengthening of the intersubunit interactions at low temperature, thereby preventing their disaggregation.

It has previously been reported that the Hill constant has a value of unity for tetracaine inhibition of firefly luciferase extract (Ueda et al., 1976) but equals 2.3 for cytochrome c

oxidase inhibition by tetracaine (Vanderkooi & Chazotte, 1982). This parameter is also greater than unity for each of the several other fragments of the mitochondrial electron-transport chain that are inhibited by local anesthetics, as evidenced by the sigmodicity of the dependence of activity on inhibitor concentration (Chazotte & Vanderkooi, 1981). Since luciferase and ATPase, both of which are lipid-free, show no inhibitor cooperativity, whereas the membrane-bound electron-transport complexes do show inhibitor cooperativity, the possibility exists that the cooperativity observed in the latter cases is the result of protein–lipid interactions.

The present results with mitochondrial ATPase may be compared with those reported previously by Cantley and Hammes (1976) for the binding of ANS and quercetin to chloroplast ATPase (CF_1). They found that ANS and quercetin compete for two tight binding sites on CF_1, which have dissociation constants of 0.03 mM for quercetin and 0.15 mM for ANS; there is in addition a larger class of about 30 much weaker sites for ANS, with dissociation constants of about 10 mM. The number of weak ANS sites (30) is of the same order of magnitude as the number of the tetracaine sites found here (~60), but the tetracaine association is stronger. It is not known whether the tetracaine and ANS sites are at similar or different loci on these proteins.

The fluorescence methods described here should be of general applicability in studying fluorophore–macromolecule interactions in cases where the fluorescence intensity of the bound and unbound ligands are both measurable. The fluorescence characteristics of tetracaine are well suited for this type of analysis.

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