Geoghegan, K. F., Galdes, A., Hanson, G., Holmquist, B., Auld, D. S., & Vallee, B. L. (1986) *Biochemistry 25*, 4669–4674.
Holmquist, B. (1986) *Methods Enzymol. 130*, 270–290.
Holmquist, B., & Vallee, B. L. (1974) *J. Biol. Chem. 249*, 4601–4607.
Holmquist, B., & Vallee, B. L. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 6216–6220.
Holmquist, B., Kaden, T. A., & Vallee, B. L. (1975) *Biochemistry 14*, 1454–1461.
Holmquist, B., Bünning, P., & Riordan, J. F. (1979) *Anal. Biochem. 95*, 540–548.
Kleeman, S. G., Keung, W. M., & Riordan, J. F. (1986) *J. Inorg. Biochem. 26*, 93–106.
Latt, S. A., & Vallee, B. L. (1971) *Biochemistry 10*, 4263–4270.
Pantoliano, M. W., Holmquist, B., & Riordan, J. F. (1984) *Biochemistry 23*, 1037–1042.
Patchett, A. A., & Cordes, E. H. (1985) *Adv. Enzymol. Relat. Areas Mol. Biol. 57*, 1–84.
Patchett, A. A., Harris, E., Tristram, E. W., Wyvratt, M. J., Wu, M. T., Taub, D., Peterson, E. R., Ikeler, T. J., ten Broeke, J., Payne, L. G., Ondeyka, D. L., Thorsett, E. D., Greenlee, W. J., Lohr, N. S., Hoffsommer, R. D., Joshua, H., Ruyle, W. V., Rothrock, J. W., Aster, S. D., Maycock, A. L., Robinson, F. M., Hirschmann, R., Sweet, C. S., Ulm, E. H., Gross, D. M., Vassil, T. C., & Stone, C. A. (1980) *Nature (London) 288*, 280–283.
Prescott, J. M., Wagner, F. W., Holmquist, B., & Vallee, B. L. (1985) *Biochemistry, 24*, 5350–5356.
Riordan, J. F., Lee, F., Pantoliano, M. W., & Holmquist, B. (1986) in *Frontiers in Bioinorganic Chemistry* (Xavier, A. V., Ed.) pp 612–621, VCH Verlagsgesellschaft, Weinhiem.
Ryan, J. W., Ryan, U. S., Schultz, D. R., Whitaker, C., Chung, A., & Dorer, F. E. (1975) *Biochem. J. 146*, 497–499.
Schäffer, A., & Auld, D. S. (1986) *Biochemistry 25*, 2476–2479.
Schatz, P. N., & McCaffery, A. J. (1970) *Q. Rev. Chem. Soc. 23*, 552–584.
Serber, R. (1932) *Phys. Rev. 41*, 489.
Shapiro, R., & Riordan, J. F. (1984) *Biochemistry 23*, 5225–5233.
Skeggs, L. T., Marsh, W. H., Kahn, J. R., & Shumway, N. P. (1954) *J. Exp. Med. 99*, 275–282.
Sytkowski, A. J., & Vallee, B. L. (1978) *Biochemistry 17*, 2850–2857.
Thiers, R. E. (1957) *Methods Biochem. Anal. 5*, 273–355.
Vallee, B. L., & Williams, R. J. P. (1968) *Proc. Natl. Acad. Sci. U.S.A. 59*, 498–505.
Vallee, B. L., & Holmquist, B. (1980) in *Methods for Determining Metal Ion Environments in Proteins: Structure and Function of Metalloproteins* (Darnall, D. W., & Wilkins, R. G., Eds.) pp 27–54, Elsevier/North-Holland, New York.
Vallee, B. L., & Galdes, A. (1984) *Adv. Enzymol. Relat. Areas Mol. Biol. 56*, 283–429.
Vallee, B. L., Galdes, A., Auld, D. S., & Riordan, J. F. (1983) *Metal Ions Biol. 5*, 25–75.
Wharton, C. W., & Szawelski, R. J. (1982) *Biochem. J. 203*, 351–360.
Yang, H. Y. T., Erdos, E. G., & Levin, Y. (1970) *Biochim. Biophys. Acta 214*, 374–376.

Temperature Dependence and Mechanism of Local Anesthetic Effects on Mitochondrial Adenosinetriphosphatase

Aaron B. Adade, Kevin L. O'Brien, and Garret Vanderkooi*
Chemistry Department, Northern Illinois University, DeKalb, Illinois 60115
Received April 17, 1987; Revised Manuscript Received July 8, 1987

ABSTRACT: Chloroform-released ATPase prepared from beef heart mitochondria is inhibited by tetracaine and dibucaine over the entire temperature range in which the enzyme is active. The temperature of maximal activity is at 60 °C in the absence of anesthetic and is shifted upward by 2–3 °C by the addition of 0.3 mM dibucaine. Local anesthetics protect ATPase from irreversible cold inactivation. The kinetics of this protective effect are analyzed by a thermodynamic model in which the associated/dissociated subunit equilibrium is shifted toward the associated state by the preferential binding of anesthetic to the associated state. The accessibility of buried sulhydryl groups to reaction with 5,5′-dithiobis(2-nitrobenzoic acid) is increased by local anesthetics; this is interpreted to mean that the anesthetics increase the conformational flexibility of the protein. It is proposed that the hydrophobic moieties of local anesthetics and related compounds bind to numerous hydrophobic sites or crevices on ATPase; this binding induces a perturbation of the protein conformation, which in turn causes a decrease of enzyme activity. This model is sufficiently general to encompass the diversity of molecules which have similar anesthetic-like effects, and since it relates to common fundamental features of protein structure, it may also be the mechanism of the nonspecific effects of these molecules on other proteins.

Local anesthetics, tricyclic antipsychotics, and related compounds inhibit mitochondrial ATPase in a partial and reversible manner (Penefsky et al., 1960; Vanderkooi et al., 1981; Palatini, 1982; Chazotte et al., 1982; Laikind et al., 1982; Saishu et al., 1983; Adade et al., 1984; Bullough et al., 1985). The membrane-bound ATPase and also the lipid-free preparations of F_1ATPase and chloroform-released ATPase are similarly inhibited by these compounds. The primary objective

0006-2960/87/0426-7297$01.50/0

of our studies has been to elucidate the mechanism by which this inhibition occurs. A major step forward was made with the recent finding, based on analysis of fluorescence data, that the ATPase–protein complex binds many molecules of tetracaine at enzyme inhibitory concentrations and that the degree of inhibition is directly proportional to the fraction of binding sites which are filled (Vanderkooi & Adade, 1986). There is a single class of about 60 sites on ATPase for tetracaine, having an intrinsic dissociation constant of 0.5 mM, this value also equals the tetracaine inhibitor constant.

A fundamental problem is to distinguish between a conformationally mediated inhibition mechanism and a specific site mechanism; a specific site mechanism has been proposed by Laikind and Allison (1983) to account for the inhibition of F_1ATPase by quinacrine, whereas Chazotte et al. (1982) have proposed a conformational mechanism for local anesthetic inhibition. It is not possible to distinguish, on the basis of kinetic data alone, between a model in which inhibition is caused by the filling of a single specific inhibitory site and a model in which inhibition is the cumulative and linearly additive result of the progressive filling of many binding sites (Vanderkooi & Adade, 1986). A specific site mechanism is conceptually simple but carries the tacit assumption that all except one (or perhaps a few) of the many binding sites are irrelevant as regards enzyme inhibition. Cumulative inhibition, on the other hand, is consistent with a conformational mechanism in which the extent of conformational change and the degree of inhibition are proportional to the number of inhibitor molecules bound. Multiple lines of evidence indicate that protein structural perturbations are caused by the same concentrations of anesthetic as give enzyme inhibition; these are considered to be evidence in favor of the hypothesis that structural perturbations are indeed closely related to the mechanism of enzyme inhibition (Chazotte et al., 1982; Kresheck et al., 1985; present paper).

In this paper, we present additional evidence that anesthetics affect the conformation and stability of chloroform-released ATPase. Anesthetics are shown to increase the reactivity of ATPase sulfhydryl groups, to protect the enzyme from cold lability, and to raise the temperature of thermal denaturation. The degree of protection from irreversible cold denaturation is shown to be directly proportional to the fraction of anesthetic binding sites which are filled. The temperature dependence of ATPase activity is reported and is quite unusual but is qualitatively similar to that previously reported for TF_1 (Yoshida et al., 1975).

Materials and Methods

Enzyme Preparation. 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). Lowe and Beechey (1982) have discussed the possible reasons that ATPase prepared by this method has a considerably lower specific activity than the F_1ATPase prepared by other methods, e.g., that of Knowles and Penefsky (1972). They determined that the chloroform-released ATPase is "at least 90% pure" as judged by polyacrylamide gel electrophoresis. Their conclusion was that the two methods of preparation yield allomorphic forms of the same enzyme which differ in certain physical and enzymatic properties and that this allomorphism, rather than the presence of gross impurity, is the reason for the difference in specific activities of the preparations. F_1ATPase and chloroform-released ATPase are both inhibited by local anesthetics.

Enzyme Assay. The continuous spectrophotometric assay using an ATP regenerating system was used for most determinations of ATPase activity (Pullman et al., 1960; Adade et al., 1984). This method could not be used at high temperature, however, because of the thermal inactivation of the coupling enzymes (lactate dehydrogenase and pyruvate kinase). A discontinuous assay based on the rate of ADP production was therefore used for high-temperature kinetic measurements. An aliquot of ATPase was added at zero time to an assay solution containing 50 mM tris(hydroxymethyl)aminomethane (Tris)–acetate, 10 mM Tris–sulfate, 0.25 M sucrose, 1 mM ethylenediaminetetraacetate (EDTA), 4 mM $MgCl_2$, and 0.5 mM ATP, pH 7.5, preequilibrated to the desired temperature. After 1.0 min, the enzyme reaction was stopped by the addition of sodium azide (final concentration 3.3 mM). The temperature of the reaction mixture was then adjusted to 25 °C and the amount of ADP was determined. This was done enzymatically by adding NADH, phosphoenolpyruvate, and lastly the coupling enzymes (lactate dehydrogenase and pyruvate kinase). The total change in NADH absorbance which occurred upon the addition of the coupling enzymes corresponds to the amount of ADP produced in the ATPase reaction. Appropriate controls were carried out to correct for any ADP which was originally present as a contaminant in the ATP, or which was produced nonenzymatically under the reaction conditions. This ADP assay procedure was both more rapid and required a smaller total degree of ATP hydrolysis than the conventional ATPase assay method based on the determination of released inorganic phosphate.

Protein concentration was determined according to Lowry et al. (1951) using bovine serum albumin as the standard [see also Muller et al. (1977)].

Sulfhydryl Group Reactivity. The rate of reaction of sulfhydryl groups with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) was determined by using the spectrophotometric assay of Habeeb (1972). A 10 mM stock solution of DTNB was prepared in 0.1 M sodium phosphate buffer at pH 8.0. An aliquot of this solution was added to ATPase to give a final DTNB concentration of 0.3 mM, and the change in absorbance was followed as a function of time at 412 nm. The number of sulfhydryl groups reacted was calculated by using an extinction coefficient of 13.6 mM^{-1} cm^{-1} (Habeeb, 1972) and an ATPase molecular weight of 360 000 as in our earlier work. [A 3% increase in the computed number of SH groups would result by using the molecular weight of 371 135 determined from the amino acid sequence (Walker et al., 1985).] The experiments reported here were done at 25 °C using ATPase at 0.6 mg/mL dissolved in its isolation buffer (10 mM Tris–sulfate, 0.25 M sucrose, and 1 mM EDTA, pH 7.5).

All biochemical reagents were obtained from Sigma.

Results

Anesthetic Effect on Sulfhydryl Group Reactivity. The presence of tetracaine or dibucaine increases the reactivity of the ATPase sulfhydryl groups with DTNB. Addition of DTNB to ATPase (in the absence of anesthetic) resulted in a rapid initial increase in absorbance at 412 nm, which was followed by a continuing slow increase in absorbance over a considerable time period. If an aliquot of tetracaine or dibucaine was added after completion of the initial rapid burst of reaction, another rapid burst of reaction occurred. Alternatively, if tetracaine or dibucaine was added to the reaction mixture before the addition of DTNB, a larger initial burst of reaction was given. These results indicate that the anesthetics increased the accessibility of SH groups for reaction with DTNB. Several control experiments were also carried out which eliminated the possible alternative explanations that the DTNB reacted with the anesthetics or that the increase

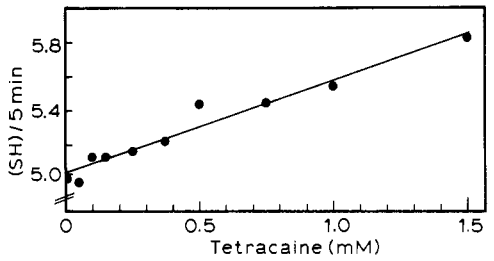

FIGURE 1: Number of sulfhydryl groups of chloroform-released ATPase which react in 5 min with DTNB, plotted as a function of the tetracaine concentration. The temperature was 25 °C.

was due to anesthetic absorbance, or to light scattering induced in the ATPase preparation by the anesthetics.

The number of SH groups per mole of ATPase which reacted in 5 min with DTNB was determined as a function of tetracaine concentration, with the tetracaine being present in the medium before the addition of DTNB. The results are plotted in Figure 1. (Similar results were obtained with dibucaine.) There is an essentially linear increase in the number of reactive SH groups over the range of tetracaine concentrations employed (up to 1.5 mM), with the net increase over this range being equivalent to about 0.8 sulfhydryl group. These observations are interpreted to mean that the anesthetics increase the amplitude of the dynamic fluctuations of the protein structure, thereby permitting a greater time-averaged accessibility of the interior sulfhydryl groups for reaction with DTNB.

Protection from Cold Inactivation. It is well-known that F_1ATPase is a cold-labile enzyme which loses its activity over a period of hours as a result of storge at 0 °C (Pullman et al., 1960). Our chloroform-released ATPase is also cold labile and therefore differs in this property from the similar material prepared by Beechey et al. (1975), which was reported to be stable in the cold. Cold lability has been attributed to the weakening of hydrophobic bonds at low temperature; by inference, hydrophobic bonds are presumed to be of relatively greater importance in ATPase and other cold-labile enzymes than in the majority of enzymes which are not cold labile (Penefsky & Warner, 1965). The study of cold lability therefore provides an experimental route for investigating the temperature dependence and the effects of additives on these hydrophobic interactions. Chaotropic salts (e.g., KI, KNO_3, KBr, $NaClO_4$, and KSCN) were shown by Penefsky and Warner (1965) to accelerate loss of ATPase activity in the cold, whereas alcohols (methanol, ethanol, ethylene glycol, and glycerol) protected from cold lability. Alkyl guanidines (Tuena de Gomez-Puyou et al., 1976) and tricyclic antipsychotics (Palatini, 1982; Bullough et al., 1985) also protect F_1ATPase from cold denaturation. We report here that the same range of concentrations of local anesthetics which cause reversible enzyme inhibition also impart protection from cold lability.

A series of tubes was set up, each containing 0.9 mL of chloroform-released ATPase at 0.6 mg/mL in the buffer used for enzyme isolation (10 mM Tris–sulfate, 0.25 M sucrose, and 1 mM EDTA, pH 7.5). To each tube was added 0.1 mL of water, or of water plus anesthetic, so as to keep the same protein concentration in all tubes. A pair of tubes without anesthetic was maintained at room temperature (22 °C), while the remaining tubes with or without anesthetics were placed on ice (0 °C). At measured time intervals, 0.01 mL was removed from each tube and added to the standard ATP regenerating assay system, for enzyme assay at 25 °C. This 100-fold dilution reduced the anesthetic concentrations to noninhibitory levels. Each determination was carried out in duplicate, and the results were averaged.

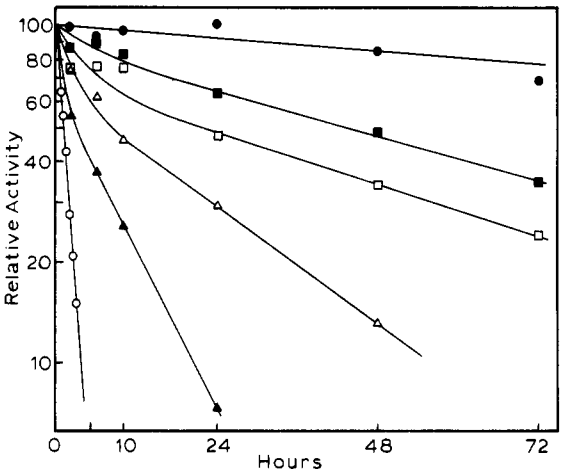

FIGURE 2: Protection of ATPase from cold inactivation by tetracaine. (●) ATPase, without tetracaine, maintained at 22 °C; (○) ATPase, without tetracaine, maintained at 0 °C. For the remaining curves, ATPase was incubated at 0 °C with the following tetracaine concentrations: (▲) 0.5 mM; (△) 1.0 mM; (□) 1.5 mM; (■) 2.0 mM. All enzyme assays were done at 25 °C after a 100-fold dilution of the tetracaine.

Table I: Kinetic and Thermodynamic Parameters for Protection of ATPase from Cold Inactivation

anesthetic	$T_{1/2}$ (h)[a]	V (h^{-1})[b]	$\Delta(\Delta G°)$[c] (cal/mol)	N[d]	$\Delta(\Delta G°)/N$[c] (cal/mol)
none	0.95	0.731			
tetracaine (0.5 mM)[e]					
0.5 mM	7.7	0.089	1140	30	38
1.0 mM	18.7	0.037	1620	40	40
1.5 mM	39.4	0.018	2010	45	45
2.0 mM	50.8	0.014	2150	48	45
dibucaine (0.12 mM)[e]					
0.3 mM	29.1	0.024	1860	43	43
0.6 mM	64.1	0.011	2290	50	46
1.2 mM	104.5	0.0066	2550	55	46
chlorpromazine (0.03 mM)[e]					
0.05 mM	17.5	0.040	1580	37	43
0.1 mM	33.3	0.021	1930	46	42
0.2 mM	84.0	0.0082	2430	52	47

[a] Half-life of ATPase activity at 0 °C, computed from the first-order rate constant. [b] First-order rate constant computed from the slopes of the linear portions of plots such as those shown in Figure 2. [c] Defined in text. [d] Number of anesthetic molecules bound per ATPase. [e] Inhibitor constant (K_I).

A semilogarithmic plot of enzyme activity as a function of incubation time is shown in Figure 2 for several tetracaine concentrations. At 0 °C in the absence of tetracaine, the plot is linear, indicative of a first-order rate process, but in the presence of tetracaine, there is curvature in the plots for the first few hours, followed by a slower first-order decay. The first-order decay rate constants were determined from the slopes of the linear portions of the semilogarithmic plots and are given in Table I. Similar data for dibucaine and chlorpromazine are also included in Table I.

Examination of the data in Table I shows that there is a close correspondence between the concentrations of the various anesthetics required for a given degree of cold protection, as measured by $T_{1/2}$, and K_I, the concentration required for half-maximal inhibition at 25 °C. It seems reasonable to suppose that there is some relationship between the mechanism of cold protection and that of enzyme inhibition, i.e., that the

binding of anesthetic molecules to the same set of sites is responsible for both of these phenomena.

Thermodynamic Analysis of Cold Protection. A simple thermodynamic scheme is adequate to account for the relative degrees of protection afforded by different concentrations of the local anesthetics. The basic mechanism will be assumed:

$$E \overset{K}{\rightleftharpoons} D \xrightarrow{k} I \quad (1)$$

E and D represent native and reversibly cold-denatured states of the enzyme, and I represents the irreversibly denatured enzyme. K is the equilibrium constant for the reversible reaction, and k is the first-order rate constant for the irreversible reaction. The formation of D is presumed to involve some degree of subunit dissociation, and I may be the result of incorrect reassociation or disulfide cross-linking of these subunits (Penefsky & Warner, 1965). The rate of conversion from D to I, which is irreversible under the conditions of our experiments, is proportional to the fraction of enzyme in the D state. The observed rate of loss of activity, V, therefore equals $k[D]$. $V/[E_t]$ may be expressed in terms of K and k, $[E_t]$ being the total enzyme concentration:

$$\frac{V}{[E_t]} = k\frac{K}{1+K} \quad (2)$$

The effect of protective agents such as local anesthetics may be explained in terms of their effects on the reversible equilibrium between the E and D states. Let K' and V' be the equilibrium constant and rate of loss of activity in the presence of a given concentration of anesthetic, respectively. The ratio of the measured decay rates (with and without anesthetic) can then be given in terms of the equilibrium constants:

$$\frac{V'}{V} = \frac{K'(1+K)}{K(1+K')} \quad (3)$$

If, as seems likely, K and K' are small under the conditions of our experiments, then $(1+K)/(1+K') << 1$, and eq 3 simplifies to $V'/V = K'/K$. The *change* in the standard free energy of the reversible denaturation reaction which occurs upon the addition of anesthetic may therefore be calculated from the measured rate constants:

$$\Delta(\Delta G°) = -RT \ln (V'/V) \quad (4)$$

Table I includes the values of $\Delta(\Delta G°)$ computed in this manner. These values, which are between 1.1 and 2.5 kcal/mol of protein, are the decrease in free energy of the E state relative to the D state which must occur upon anesthetic binding in order to account for the decreased rate of irreversible loss of activity.

This analysis can be taken one step further by calculating the average free energy change per bound anesthetic molecule. The average number of bound tetracaine molecules may be calculated as a function of the tetracaine concentration by using the dissociation constant and number of binding sites determined previously (Vanderkooi & Adade, 1986). [Those values were determined at 25 °C, but there does not appear to be a strong temperature dependence for the dissociation constant, as indicated by the fact that the degree of inhibition does not greatly change with temperature (Chazotte et al., 1982; present work).] For dibucaine and chlorpromazine, the number of bound molecules may likewise be estimated if their dissociation constants are assumed to equal their respective inhibitor constants (as is the case for tetracaine) and by assuming the same number of binding sites (60) as was determined for tetracaine. Table I includes the numbers of bound

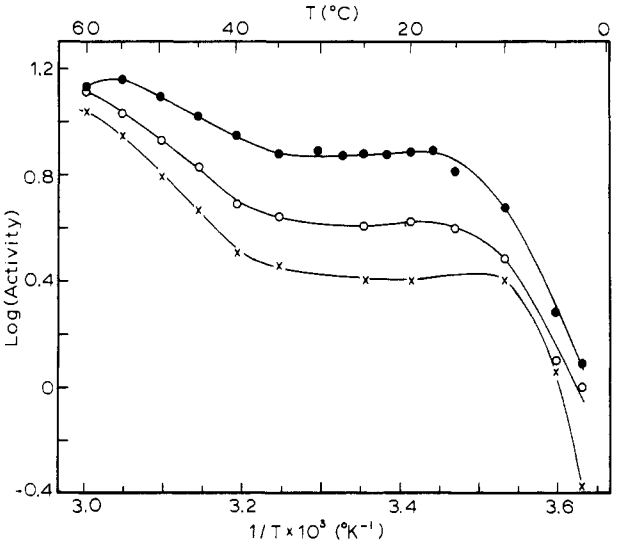

FIGURE 3: Arrhenius plot of chloroform-released ATPase. The activity was determined by using the ATP regenerating coupled enzyme assay method (Pullman et al., 1960). (○) No anesthetic; (●) 0.15 mM dibucaine; (×) 0.30 mM dibucaine.

anesthetic molecules (N) as calculated with these assumptions. The mean free energy change per bound anesthetic molecule, $\Delta(\Delta G°)/N$, can then be calculated, and the values are given in the last column of Table I. These values are remarkably similar, being between 38 and 47 cal/mol of bound anesthetic for all concentrations of the three anesthetics. They are also small, being less than 1% of the standard free energy of binding of the anesthetics to the protein, as calculated from the dissociation constants. Of course, one cannot differentiate between a change in the free energy difference per molecule and a change in the average number of molecules bound; all that is required is that the product of these quantities equals $\Delta(\Delta G°)$. The constancy of $\Delta(\Delta G°)/N$ shows, however, that in either case $\Delta(\Delta G°)$ is proportional to the number of sites filled.

In molecular terms, the weakening of the anesthetic–protein interactions (increase in free energy) on going from the E to the D state may be understood if some of the anesthetic binding sites lie between protein subunits, since dissociation of the subunits would cause a disruption of these sites. This picture is not inconsistent with that given to account for the greater reactivity of sulfhydryl groups in the presence of anesthetic. Insertion of anesthetic molecules in crevices between subunits would be expected to increase the dynamic fluctuations or breathing motions of the protein as a whole, thus increasing sulfhydryl group accessibility (a kinetic effect), but at the same time may cause a shift in the association/dissociation equilibrium toward the associated state as a result of the stronger interactions with the associated form (a thermodynamic effect).

Temperature Dependence of ATPase Activity. The ATPase activity was determined as a function of temperature in the absence and presence of dibucaine. The results of these measurements are shown in Figures 3 and 4. The ATP regenerating, coupled enzyme assay system was used for the experiment shown in Figure 3, but the activities reported in Figure 4 were measured by using the ADP release assay described under Materials and Methods. The apparent activities determined by the latter method are always smaller than those given by the coupled enzyme assay, evidently because of inhibition of the ATPase reaction by the hydrolysis products.

For the experiments shown in Figures 3 and 4, the buffer was adjusted to pH 7.5 at 25 °C with no further adjustments

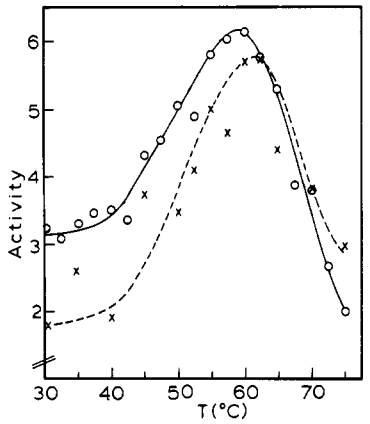

FIGURE 4: High-temperature activity of ATPase. The activity was determined by using the discontinuous ADP assay procedure described under Materials and Methods. (○) No anesthetic; (×) 0.3 mM dibucaine.

being made to compensate for changes of pH with temperature. This was done in order to facilitate the comparison of the Arrhenius plot with the results of our previous differential scanning calorimetry measurements, in which the adjustment of the pH as a function of temperature was of course not possible (Kresheck et al., 1985; see Discussion). In a separate experiment (not shown), a series of buffers was prepared, one for each 5 °C temperature interval, with the pH being adjusted to 7.5 at each temperature by using a Corning Model 155 pH meter equipped with an automatic temperature compensator. An Arrhenius plot of ATPase activity was then determined by using these buffers. The result obtained was very similar to that shown in Figure 3. This was not unexpected considering the rather mild dependence of activity on pH for the range in question (Adade et al., 1984).

It can be seen from Figure 3 that ATPase has a remarkable temperature dependence. The activity rises rapidly between 2.5 and 17 °C, but between 20 and 35 °C, the activity is virtually constant. Above 35 °C, the activity increases again, reaching a maximum at 60 °C, as seen in Figure 4. The apparent activation energy is 19 kcal/mol between 2.5 and 17 °C, and 6.3 kcal/mol between 35 and 55 °C, but it is meaningless to calculate an activation energy for the range between 20 and 35 °C since the slope is essentially zero.

Dibucaine inhibits the ATPase at all temperatures (Figure 3). The general shape of the temperature–activity curve remains the same in the presence as in the absence of dibucaine, and the relative degree of inhibition is seen to be quite similar over most of the temperature range, in agreement with the report of Chazotte et al. (1982). Dibucaine shifts the temperature of maximal activity upward by 2–3 °C, as can be seen in Figure 4. This is equivalent to stabilizing the enzyme against heat denaturation. Enzyme inhibition by dibucaine still occurs at temperatures above 60 °C, however, as demonstrated by the inhibiting effects of higher dibucaine concentrations than reported in Figure 4.

The temperature dependence determined here for ATPase prepared from beef heart mitochondria is qualitatively similar to that of TF_1, the ATPase isolated from a thermophilic bacterium (Yoshida et al., 1975). TF_1 also shows a sharp rise in activity at low temperature, a local maximum at 20 °C which is followed by a region of lower activity between 20 and 40 °C, and a peak of maximal activity at 75 °C. It is interesting to note that the temperature optimum for this thermophile enzyme is only 15 °C higher than that found here for the mammalian enzyme; both are uncommonly stable with respect to heat.

The Arrhenius plot in Figure 3 is similar to that given by Harris et al. (1981) for F_1ATPase. They reported a break at 18 °C and a plateau of essentially zero slope above that temperature, but their plot did not go above 37 °C and hence did not show the high temperature maximum. Kerimov et al. (1978) and Dorgan et al. (1984) likewise found a change in slope at 18–20 °C for F_1ATPase, but Solani and Bertoli (1981) showed an Arrhenius plot for chloroform-released ATPase which was linear over the entire temperature range employed (8–33 °C).

Discussion

Sulfhydryl Group Reactivity. Senior (1973, 1975) reported that there are eight sulfhydryl groups and two disulfide bonds in F_1ATPase, with two of the sulfhydryl groups being readily accessible for reaction with DTNB. Treatment with sodium dodecyl sulfate or 6 M guanidine hydrochloride resulted in subunit dissociation and exposure of all of the sulfhydryl groups. [Walker et al. (1985) more recently reported a total of eight half-cystine residues in F_1ATPase.] Penefsky and Warner (1965) found that the addition of chaotropic salts (e.g., 0.1 M KNO_3) caused an increase in the number of sulfhydryl groups that reacted with DTNB at 25 °C. The effect of anesthetics on sulfhydryl reactivity is superficially similar to that of the chaotropic salts, in that both types of compounds appear to make the ATPase structure less rigid and to increase the accessibility of buried sulfhydryl groups. The mechanisms of these effects must be quite different, however. The chaotropic salts are well-known for their ability to decrease the conformational stability of proteins (Arakawa & Timasheff, 1982), evidently by causing an increase in the solubilizing power of the medium. At low temperature, the subunit dissociation reaction of ATPase is strongly favored by these salts, and it is probably the same tendency at room temperature which results in the increased reactivity of the buried sulfhydryl groups. The anesthetics, on the other hand, evidently perturb the structure by penetrating into hydrophobic pockets, which increases the accessibility of the sulfhydryl groups, but without causing dissociation into subunits.

Protection from Cold Lability. It was shown that the decrease in rate of irreversible loss of activity at 0 °C is directly related to the number of moles of anesthetic bound per mole of ATPase, and this was explained in terms of preferential binding to the associated state. A similar binding mechanism probably also accounts for the protection from cold denaturation by alkyl guanidines (Tuena de Gomez-Puyou et al., 1978) and other tricyclic antipsychotics (Palatini, 1982; Bullough et al., 1985), but an indirect mechanism involving the properties of the medium is probably responsible for the protection from cold denaturation by alcohols. The concentrations of alcohols required for protection are in the molar range, as compared to the millimolar range for anesthetics (Penefsky & Warner, 1965). The studies of Lee and Timasheff (1977), and Na and Timasheff (1981), on the effects of glycerol on tubulin polymerization also seem relevant to the understanding of alcohol effects on ATPase. They showed that tubulin is preferentially hydrated in the presence of glycerol, which is equivalent to saying that glycerol is excluded from the immediate vicinity of the protein. This exclusion results in thermodynamic destabilization of the system. Since the degree of exclusion and destabilization is proportional to the exposed surface area of the protein, tubulin polymerization (which decreases surface area) is favored by the presence of glycerol. The protection of ATPase from dissociation in the cold by glycerol and other alcohols probably follows an analogous mechanism.

Temperature Dependence of ATPase Activity. The complex form of the Arrhenius plot of ATPase is most likely the result of temperature-dependent conformational transitions between states of different intrinsic activity and activation energy. At least two conformational states must be assumed in order to account for the three slopes between 2.5 and 60 °C (Figure 3). The activation energy is 6.3 kcal/mol above 35 °C and 19 kcal/mol below 17 °C. These values may correspond to the activation energies of high- and low-temperature forms of the enzyme. One might be tempted to suggest that yet another conformational state exists between 20 and 35 °C but the apparent zero activation energy in this range is physically unrealistic; it is more plausible to suppose that in the 20–35 °C range the enzyme undergoes a conformational transition between low- and high-temperature states, with the observed activity in this temperature range being the sum of the activities of the two components. The qualitatively similar inflexion in the Arrhenius plot of trypsin has been analyzed mathematically by using a two-state model of this type (Talski, 1971). Since at low temperature the apparent activation energy is 19 kcal/mol, but the same chemical reaction occurs at high temperature with an activation energy of only 6.3 kcal/mol, it may be that the apparent enzyme activation energy at low temperature includes the enthalpy of an endothermic conformational transition, so that the observed value of 19 kcal/mol is the sum of a conformational enthalpy term and a true activation energy. This hypothesized conformational transition may be the same as, or is in some way related to, the reversible cold denaturation reaction already described.

Some physical evidence has appeared which supports the proposal that conformational changes occur in the ATPase as a function of temperature. Kerimov et al. (1978) found that the electron spin resonance correlation times of two spin-labels covalently bound to F_1ATPase showed changes in slope as a function of temperature at 18–20 °C and at 34–37 °C, and they related these changes to the Arrhenius plot breaks which they observed in the same temperature ranges. The intrinsic tryptophan fluorescence of oligomycin-sensitive ATPase likewise showed changes in the slope of a plot of fluorescence intensity vs temperature between 18 and 31 °C (Parenti-Castelli et al., 1983). The later finding is not necessarily applicable to the present discussion, however, on account of the presence of lipid and the large number of protein subunits in oligomycin-sensitive ATPase (F_0F_1ATPase) as compared to F_1ATPase.

The stabilizing effect of dibucaine on ATPase observed at high temperature was unexpected. We previously reported that the temperature maximum of cytochrome *c* oxidase (Chazotte & Vanderkooi, 1981; Vanderkooi & Chazotte, 1982) was shifted to a significantly lower temperature by anesthetics, and we also found that the endothermic transition of the ATPase as measured by differential scanning calorimetry (DSC) was shifted to lower temperatures by tetracaine and dibucaine (Kresheck et al., 1985). The DSC peak shifted from 80.5 to 71.7 °C upon the addition of 0.3 mM dibucaine, but this same concentration of dibucaine caused the temperature of maximal enzyme activity to shift upward by 2–3 °C, from 60 °C. It seems unlikely that these differences in thermal behavior can be attributed to the inevitable difference in protein concentration or the differing composition of the media employed in the DSC and kinetic experiments; it is more plausible that different conformational events are being observed by the two methods. We suggest that enzyme activity decreases above 60 °C as a result of quaternary conformational changes but that denaturation of the tertiary structure occurs at 80 °C (or lower in the presence of anesthetics). If this is correct, it means that local anesthetics stabilize the native quaternary structure both in the cold and at high temperature but destabilize the tertiary structure with respect to thermal denaturation.

If the integrity of the quaternary structure is lost at a lower temperature than the major endothermic transition observed by DSC, then the size of the cooperative unit which melts in the DSC experiment might be expected to be smaller than that of the entire multisubunit complex. This is indeed the case. Kresheck et al. (1985) reported that ΔH_{cal}, the calorimetric heat of denaturation of ATPase, is 6800 ± 700 kcal/mol but that the van't Hoff heat (ΔH_{vh}), computed from the peak shape, is only 470 kcal/mol. The ratio of these two heats should be close to unity if the entire protein melts as a single cooperative unit, but since the measured ratio is about 1:14, it indicates that the size of the cooperative unit is only about $^1/_{14}$th of the entire protein, corresponding to a molecular weight of 25 000. This is the same order of magnitude as the size of the ATPase subunits.

Two denaturational steps are proposed here, the first in which enzyme activity is lost as a result of the breakdown of quaternary structure and the second in which the tertiary structure is lost. There is only a single peak in the DSC trace, however (Kresheck et al., 1985). An explanation for this apparent inconsistency can be found in the relative magnitudes of the heats involved. The denaturational heat, computed from an Arrhenius plot of the enzyme activity data, is only 30 kcal/mol, whereas the calorimetric heat of the 80 °C transition is 6800 ± 700 kcal/mol. A calorimetric peak at 60 °C of only 30 kcal/mol would therefore have a peak area of 0.4% of the area of the major peak and would not be visible with the experimental conditions employed.

Conclusions. It has proved possible to give self-consistent interpretations for the several diverse observations on the effects of local anesthetics on ATPase, in terms of a model in which the binding of the anesthetic molecules to nonspecific hydrophobic sites causes tertiary and quaternary changes in the structure and stability of the protein, in a temperature-dependent manner. This model readily accounts for the observed partial inhibition of enzyme activity, since it is quite plausible to assume that the moderate structural changes which result from the binding may also alter the shape and efficacy of the active site. There is therefore no necessity to invoke specific site binding, in addition to the demonstrated nonspecific binding, in order to account for enzyme inhibition. The nonspecific nature of the binding is implied both by the large number of sites which have equivalent intrinsic dissociation constants, as determined for tetracaine (Vanderkooi & Adade, 1986), and also by the many kinds of molecules which have similar, although not identical, effects (Chazotte et al., 1982; Adade et al., 1984; Palatini, 1982; Tuena de Gomez-Puyou et al., 1976). (We have recently shown that the antimalarial drug chloroquine, which shares common structural features with the local anesthetics, also gives partial inhibition of ATPase. The concentration required for half-maximal inhibition is 0.6 mM.)

Since the explanations given here for the various anesthetic effects on ATPase primarily relate to fundamental features of protein structure, the same types of effects may be expected to occur in other proteins. This model may therefore have general applicability to the nonspecific effects of local anesthetics and related compounds on other enzymes as well.

Registry No. ATPase, 9000-83-3; tetracaine, 94-24-6; dibucaine, 85-79-0; chlorpromazine, 50-53-3.

REFERENCES

Adade, A. D., Chignell, D., & Vanderkooi, G. (1984) *J. Bioenerg. Biomembr. 16*, 353–363.

Arakawa, T., & Timasheff, S. N. (1982) *Biochemistry 21*, 6545–6552.

Beechey, R. B., Hubbard, S. A., Linnett, P. E., Mitchell, A. D., & Munn, E. A. (1975) *Biochem. J. 148*, 533–537.

Bullough, D. A., Kwan, M., Laikind, P. K., Yoshida, M., & Allison, W. S. (1985) *Arch. Biochem. Biophys. 236*, 567–575.

Chazotte, B., & Vanderkooi, G. (1981) *Biochim. Biophys. Acta 636*, 153–161.

Chazotte, B., Vanderkooi, G., & Chignell, D. (1982) *Biochim. Biophys. Acta 680*, 310–316.

Dorgan, L. J., Urbauer, J. L., & Schuster, S. M. (1984) *J. Biol. Chem. 259*, 2816–2821.

Gekko, K., & Timasheff, S. N. (1981) *Biochemistry 20*, 4667–4676.

Habeeb, A. F. S. A. (1972) *Methods Enzymol. 25*, 457–464.

Harris, D. A., Dall-Larsen, T., & Klungsøyr, L. (1981) *Biochim. Biophys. Acta 635*, 421–428.

Kerimov, T. M., Mil'grom, Y. M., Kozlov, I. A., & Runge, E. K. (1978) *Biokhimiya (Moscow) 43*, 1525–1531.

Knowles, A. F., & Penefsky, H. S. (1972) *J. Biol. Chem. 247*, 6617–6623.

Kresheck, G. C., Adade, A. B., & Vanderkooi, G. (1985) *Biochemistry 24*, 1715–1719.

Laikind, P. K., & Allison, W. S. (1983) *J. Biol. Chem. 258*, 11700–11704.

Laikind, P. K., Goldenberg, T. M., & Allison, W. S. (1982) *Biochem. Biophys. Res. Commun. 109*, 423–427.

Lee, J. C., & Timasheff, S. N. (1977) *Biochemistry 16*, 1754–1764.

Linnett, P. E., Mitchell, A. D., Partis, M. D., & Beechey, R. B. (1979) *Methods Enzymol. 55*, 337–343.

Lowe, P. N., & Beechey, R. B. (1982) *Biochem. Int. 4*, 9–15.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Muller, J. L. M., Rosing, J., & Slater, E. C. (1977) *Biochim. Biophys. Acta 462*, 422–437.

Na, G. C., & Timasheff, S. N. (1981) *J. Mol. Biol. 151*, 165–178.

Palatini, P. (1982) *Mol. Pharmacol. 21*, 415–421.

Parenti-Castelli, G., Baracca, A., Fato, R., & Rabbi, A. (1983) *Biochem. Biophys. Res. Commun. 111*, 366–372.

Penefsky, H. S., & Warner, R. C. (1965) *J. Biol. Chem. 240*, 4694–4702.

Penefsky, H. S., Pullman, M. E., Datta, A., & Racker, E. (1960) *J. Biol. Chem. 235*, 3330–3336.

Pullman, M. E., Penefsky, H. S., Datta, A., & Racker, E. (1960) *J. Biol. Chem. 235*, 3322–3329.

Saishu, T., Kagawa, Y., & Shimizu, R. (1983) *Biochem. Biophys. Res. Commun. 112*, 822–826.

Senior, A. E. (1973) *Biochemistry 12*, 3622–3627.

Senior, A. E. (1975) *Biochemistry 14*, 660–664.

Solani, G., & Bertoli, E. (1981) *FEBS Lett. 132*, 127–128.

Talski, G. (1971) *Angew. Chem., Int. Ed. Engl. 10*, 548–554.

Tuena de Gomez-Puyou, M., Gomez-Puyou, A., & Beigel, M. (1976) *Arch. Biochem. Biophys. 173*, 326–331.

Vanderkooi, G., & Chazotte, B. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 3749–3753.

Vanderkooi, G., & Adade, A. B. (1986) *Biochemistry 25*, 7118–7124.

Vanderkooi, G., Shaw, J., Storms, C., Vennerstrom, R., & Chignell, D. (1981) *Biochim. Biophys. Acta 635*, 200–203.

Walker, J. E., Fearnly, I. M., Gay, N. J., Gibson, B. W., Northrop, F. D., Powell, S. J., Runswick, M. J., Saraste, M., & Tybulewicz, V. L. J. (1985) *J. Mol. Biol. 184*, 677–701.

Yoshida, M., Sone, N., Hirata, H., & Kagawa, Y. (1975) *J. Biol. Chem. 250*, 7910–7916.