Differential Scanning Calorimetry Study of Local Anesthetic Effects on F_1ATPase and Submitochondrial Particles†

Gordon C. Kresheck,* Aaron B. Adade, and Garret Vanderkooi*
Department of Chemistry, Northern Illinois University, DeKalb, Illinois 60115
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ABSTRACT: The differential scanning calorimetry trace of F_1ATPase, prepared from beef heart submitochondrial particles, has a single sharp endothermic transition at 80.5 ± 1.0 °C and a half-height peak width of 2.0 ± 0.2°. The transition enthalpy is 19 ± 2 cal/g of protein. Submitochondrial particles (SMP) have a similar peak at 75.1 ± 0.5 °C with a half-height peak width of 1.8 ± 0.1° and an enthalpy of 5 ± 1 cal/g of SMP protein. The SMP transition is provisionally identified as being due to membrane-bound F_1ATPase. Tetracaine and dibucaine cause these transitions to shift to lower temperatures; addition of 0.3 mM dibucaine gives peaks at 71.7 and 64.9 °C for F_1ATPase and SMP, respectively, and 1.0 mM tetracaine gives peaks at 70.0 and 60.5 °C for F_1ATPase and SMP, respectively. These anesthetic concentrations also give appreciable inhibition of enzyme activity at 25 °C. We conclude that the local anesthetics induce conformational alterations in the F_1ATPase–protein complex which result both in enzyme inhibition and in the lowering of the thermal denaturation transition temperature.

F_1ATPase is an extrinsic membrane protein which is associated in vivo with the inner mitochondrial membrane, being a major part of the ATP synthetase complex. Extraction from the membrane yields a water-soluble protein having ATPase activity, which is free of lipid. The enzyme prepared from beef heart mitochondria has a molecular weight of about 360 000 and contains five different subunits, the largest of which is 53 000 daltons (Senior, 1979). One of the remarkable features of this enzyme is its cold lability (Pullman et al., 1960); it is stable for days at room temperature, but enzymic activity is quickly lost if the solution is placed on ice.

We have found that F_1ATPase is very sensitive to inhibition by tertiary amine local anesthetics (Vanderkooi et al., 1981; Chazotte et al., 1982). The inhibitory potencies of these agents are proportional to their octanol/water partition coefficients, just as is the case for nerve blocking and for the inhibition of various membrane-bound enzyme activities by these compounds (Seeman, 1972; Chazotte & Vanderkooi, 1981). Chlorpromazine and other tricyclic antipsychotics or tranquilizers also inhibit F_1ATPase in an apparently similar manner to the local anesthetics; these have been studied by Penefsky et al. (1960), who first reported the inhibition of F_1ATPase by chlorpromazine, and by Palatini (1982).

We are attempting to determine the mechanism by which local anesthetics inhibit F_1ATPase, both because this enzyme is itself intrinsically interesting and significant and also because some aspects of the mechanism involved here may apply as well to the effects of anesthetics on nerves and other membrane enzymes. The interpretation of mechanistic studies on membrane-bound enzymes or transport systems is made difficult and uncertain by the presence of lipid, but with F_1ATPase all observed effects can safely be attributed to anesthetic interactions with the protein since no lipid is present.

Kinetic analysis of the effects of local anesthetics on F_1ATPase activity indicates that these agents act as partial, noncompetitive, reversible inhibitors (Chazotte et al., 1982; Adade et al., 1984). We have proposed that the inhibition is a result of a conformational perturbation of the protein, probably at the quaternary level of organization (Chazotte et al., 1982). This proposal was based in part on the observations that 1 mM tetracaine caused changes in the schlieren pattern of F_1ATPase in the analytical ultracentrifuge and also caused the production of multiple bands in gel electrophoresis. This proposal is also compatible with the partial noncompetitive nature of the inhibition. Our work was carried out with F_1ATPase prepared from beef heart mitochondria by the chloroform method of Beechey et al. (1975), but it has been confirmed by Laikind et al. (1982), and Laikind & Allison (1983), that F_1ATPase prepared by the conventional method of Knowles & Penefsky (1972) is likewise inhibited by local anesthetics.

Results of differential scanning calorimetry (DSC) studies on the thermal denaturation of F_1ATPase are presented in this paper. We found that local anesthetics, in the same concentration range as required for enzyme inhibition, cause a dramatic decrease in the transition temperature for thermal denaturation of F_1ATPase (T_m). DSC measurements on intact submitochondrial particles (SMP) gave a transition in the same temperature range as the F_1ATPase denaturational transition; the T_m for this transition is also depressed by local anesthetics. We therefore have provisionally identified the SMP peak as being due to the denaturation of membrane-associated F_1ATPase.

Materials and Methods

Preparations. Frozen beef heart mitochondria were obtained from The Institute for Enzyme Research, Madison, WI. Submitochondrial particles were prepared according to procedure II of Gregg (1967). The SMP were stored until use in small aliquots in 0.25 M sucrose at a concentration of 33 mg of protein/mL at −20 °C.

The chloroform extraction method of Beechey et al. (1975) was used to prepare F_1ATPase from SMP. The SMP were diluted in sucrose buffer [0.25 M sucrose, 10 mM tris(hydroxymethyl)aminomethane–sulfate (Tris–sulfate), and 1 mM ethylenediaminetetraacetic acid (EDTA), pH 7.4] to a protein concentration of 8 mg/mL, after which 1 part analytical reagent-grade chloroform was added per 2 parts SMP suspension. The mixture was agitated vigorously for 30 s after which the emulsion was broken by centrifugation for 15 min

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at 600*g*. The aqueous upper phase was recentrifuged for 40 min at 39000*g*, and the supernatant, which contained the F_1ATPase, was used without further purification. The protein concentration of this solution was 0.8–0.9 mg/mL, determined by using bovine serum albumin as the standard (Lowry et al., 1951; Muller et al., 1977). Polyacrylamide gel electrophoresis of this material showed a single prominent band (Chazotte et al., 1982). Under optimal assay conditions, the specific activity was 14 μmol of ATP hydrolyzed min^{-1} (mg of protein)$^{-1}$ at 25 °C; this compares favorably with values obtained in other laboratories with the same type of preparation, as summarized by Linnett et al. (1979).

The possibility of phospholipid contamination in the F_1ATPase preparation was checked by extraction with chloroform/methanol (2:1 v/v), followed by thin-layer chromatography on silica gel plates using chloroform/methanol/water (65:25:4 v/v) as the developing solvent. No evidence for the presence of phospholipid was found; control experiments indicated that 1 or 2 mol of phospholipid per mole of F_1ATPase could have been detected. This result is in agreement with a previous report (Spitzberg & Blair, 1977).

Enzyme Activity. The ATPase activity of F_1ATPase and SMP was measured by using an ATP-regenerating system similar to that described by Pullman et al. (1960). The assay system consisted of 0.25 M sucrose, 10 mM Tris–sulfate, 1 mM EDTA, 4 mM $MgCl_2$, 2.5 mM phosphoenolpyruvate, 0.5 mM ATP, 0.35 mM NADH, 8 units of lactate dehydrogenase, 6.5 units of pyruvate kinase (the latter two enzymes being added together as an aliquot of Sigma type I lactate dehydrogenase), an aliquot of F_1ATPase solution, and anesthetics as desired. All assays were at pH 7.4 at 25 °C. The first three components of the assay system replaced the 50 mM Tris–acetate buffer used in our previous work (Chazotte et al., 1982) for consistency with the buffer system used in the DSC measurements.

DSC Measurements. The DSC studies were carried out on an MC-1 differential scanning calorimeter (MicroCal, Amherst, MA) which was interfaced to an IBM 9000 computer system. The sample volume was 0.61 mL. Data were collected between 5 and 95 °C at a heating rate of 1.18 °C/min. The digitalized data were stored in the computer (usually 728 points for each run), which were subsequently recalled for processing and computation of the thermodynamic quantities. Electrical calibration was used to convert the measured voltage signals to calories. The transition temperatures (T_m), transition enthalpy (ΔH_d), maximum excess apparent specific heat (C_{max}), and transition width at half peak height ($T_{1/2}$) were determined for each run. ΔH_d is proportional to the area under the peak, and C_{max} is proportional to the peak height. Peak areas were determined by numerical integration of the digitalized data. Since there was no significant evidence of a change in heat capacity accompanying the transition for either F_1ATPase or SMP, the base line of the peak was taken as the straight line connecting the observed base lines below and above the transition for integration purposes. The transition temperatures and enthalpies of unsonicated samples of dimyristoylphosphatidylcholine and dipalmitoylphosphatidylcholine were determined by these methods and were found to agree within experimental error with published values (Gaber & Sheridan, 1982).

The van't Hoff transition enthalpy was estimated on the assumption of a two-state process using the following equation (Privalov, 1979):

$$\Delta H_{vh} = 4R(T_m + 273.15)^2 \frac{C_{max}}{\Delta H_d} \quad (1)$$

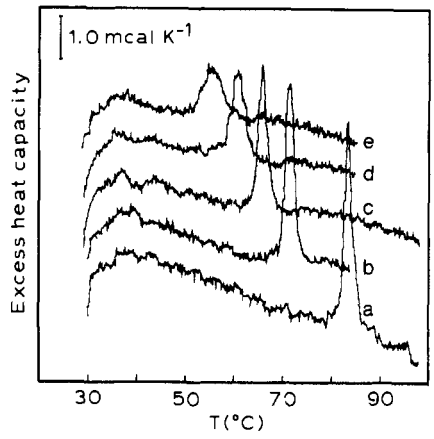

FIGURE 1: Differential scanning calorimetry traces of F_1ATPase in 0.25 M sucrose, 10 mM Tris–sulfate, and 1 mM EDTA, pH 7.4. Trace a, F_1ATPase alone; traces b, c, d, and e, dibucaine concentrations of 0.3, 0.6, 1.0, and 1.5 mM, respectively. The protein concentration was 0.77 mg/mL.

The DSC measurements were made on F_1ATPase or SMP in the sucrose buffer used in the F_1ATPase preparation, diluted 10% by the addition either of water or of an aqueous stock solution of tetracaine hydrochloride or dibucaine hydrochloride. The final protein concentration was 0.7–0.8 mg/mL for F_1ATPase and 2–3 mg/mL for SMP. F_1ATPase was used within 24 h of preparation, and the SMP were used on the same day as prepared. SMP were transferred into the sucrose buffer by centrifugation; 1 mL of frozen SMP was thawed, diluted to 10 mL with sucrose buffer, pelleted by centrifugation for 25 min at 90000*g*, and resuspended by homogenization to the desired final concentration with the same sucrose buffer. The SMP protein concentration was determined on the final solution (Lowry et al., 1951).

RESULTS

DSC Measurements on F_1ATPase. A single sharp endothermic transition was found for F_1ATPase at 80.5 ± 1 °C, which was attributed to the thermal denaturation of the protein. This peak disappeared when the sample was cooled and reheated in the calorimeter, showing that the thermal denaturation of the enzyme was irreversible under the conditions employed. The DSC traces for F_1ATPase alone and in the presence of several concentrations of dibucaine are shown in Figure 1. Thermodynamic parameters derived from these curves are given in Table I.

Addition of either tetracaine or dibucaine to F_1ATPase caused the endothermic transition to shift to lower temperatures, as can be seen from the representative traces for dibucaine shown in Figure 1. For example, 0.3 mM dibucaine, or 1.0 mM tetracaine, caused a lowering of T_m by 9–10 °C, with little or no peak broadening or decrease in peak height (see Table I). Higher concentrations of both agents did cause $T_{1/2}$ increase, C_{max} to decrease, and ΔH_d to decrease (Table I). The ability of dibucaine to have a given effect at lower concentrations that tetracaine was also observed in our earlier enzyme inhibition studies (Chazotte et al., 1982).

DSC Measurements on SMP. Figure 2 shows DSC traces of SMP in the absence and presence of dibucaine. SMP alone display a sharp thermally irreversible endothermic peak at 75 °C which shifts to lower temperatures as dibucaine or tetracaine is added. The numerical parameters for these measurements are included in Table I. On the basis of the similar transition temperatures (75 and 80 °C) and half-height peak widths (1.8° and 2.0°) of SMP and F_1ATPase, respectively, as well as their mutual sensitivity to being shifted to lower

Table I: Calorimetric Parameters for the Thermal Transition of F_1ATPase and SMP as a Function of Tetracaine and Dibucaine Concentrations

system[a]	anesthetic	concn (mM)[b]	T_m (°C)	T (°C)	C_{max} (cal K^{-1} g^{-1})	ΔH_d (cal/g)	ΔH_{cal} (kcal/mol)[c]	ΔH_{vh} (kcal/mol)[d]
F_1ATPase	tetracaine	0 (6)	80.5 ● 1.0	2.0 ± 0.2	9 ± 1	19 ± 2	6800 ± 700	470
		0.5	76.1	2.2	10.0	23	8300	420
		1.0	70.0	1.9	8.8	18	6500	460
		2.0	64.5	2.8	6.1	17	6100	330
		2.5	61.6	2.5	5.3	14	5000	340
	dibucaine	0.3	71.7	2.1	8.7	17	6100	480
		0.6	64.4	1.9	7.0	16	5800	400
		1.0	59.2	2.8	4.2	13	4700	280
		1.5	52.9	4.1	2.3	9.5	3400	200
SMP	tetracaine	0 (3)	75.1 ± 0.5	1.8 ± 0.1	3 ± 1	5 ± 1		580
		0.5	66.3	1.8	2.4	3.2		690
		1.0	60.5	1.9	1.5	3.1		430
		1.5	51.0	4.8	0.7	3.8		150
	dibucaine	0.15	69.0	2.1	3.6	8.2		400
		0.3	64.9	2.2	2.8	6.0		420
		0.45	62.5	2.4	1.8	2.4		670

[a] F_1ATPase and SMP were in sucrose buffer (0.25 M sucrose, 10 mM Tris-HCl, and 1.0 mM EDTA, pH 7.4). [b] The values in parentheses are the number of determinations. Where not shown, a single measurement was made under the conditions described. [c] Computed from ΔH_d, assuming a molecular weight of 360 000. [d] ΔH_{vh} was computed by using eq 1.

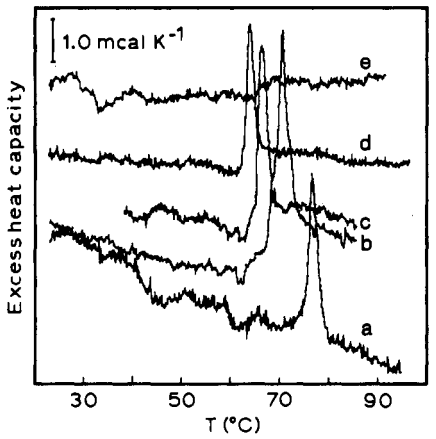

FIGURE 2: Differential scanning calorimetry traces of submitochondrial particles prepared from beef heart suspended in 0.25 M sucrose, 10 mM Tris–sulfate, and 1 mM EDTA, pH 7.4. Trace a, SMP alone; traces b, c, d, and e, dibucaine concentrations of 0.15, 0.3, 0.45, and 0.6 mM, respectively; no distinct peak is present in trace e. The protein concentration was 3.2 mg/mL for trace a, 2.3 mg/mL for traces b and c, 2.8 mg/mL for trace d, and 2.0 mg/mL for trace e.

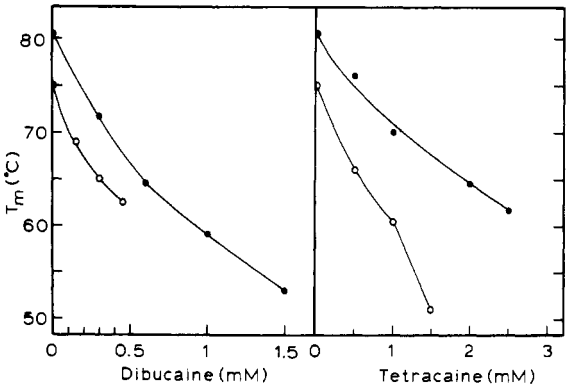

FIGURE 3: Dependence of the endothermic transition temperature (T_m) of F_1ATPase and SMP on the dibucaine and tetracaine concentrations: (●) F_1ATPase; (○) SMP. Other conditions are as described in the text.

temperatures by anesthetics, we provisionally conclude that the 75 °C SMP transition is due to membrane-bound F_1ATPase.

The dependence of the T_m values of F_1ATPase and SMP on the dibucaine and tetracaine concentrations are compared in Figure 3. It is seen that for dibucaine concentrations up to 0.45 mM and tetracaine concentrations up to 1.0 mM, the change in T_m with anesthetic concentration is similar for the two systems. Differences in behavior are observed at higher concentrations, however. Dibucaine concentrations of 0.6 mM and above, and tetracaine at 2.0 mM, caused the disappearance of the endothermic transition of SMP; these same concentrations caused the broadening but not the disappearance of the F_1ATPase peak. It appears that anesthetics affect the SMP transition in a two-step process; the first is similar to that observed in F_1ATPase but the second, which results in the abolition of the endothermic peak, only occurs in SMP.

The reversibility of the tetracaine and dibucaine effects on SMP was demonstrated by incubating SMP samples in the presence of 0.3 mM dibucaine or 1.0 mM tetracaine for 30 min at 4 °C, after which the SMP were transferred to fresh buffer by centrifugation. The T_m values of samples treated in this manner were 72.6 and 74.3 °C, respectively, for the samples which had been exposed to dibucaine or tetracaine, as compared to 75.1 ± 0.5 °C for samples which had not been so exposed. The peak widths were also similar (2.2° for both anesthetics, as compared to 1.8° for unexposed SMP). These small differences might be accounted for by residual anesthetic in the particles.

No other peaks or troughs were reproducibly present in the DSC traces of SMP in the range between 20 and 90 °C. [Hackenbrock (1977) has shown that the major membrane lipid transition of mitochondria occurs near 0 °C.] With freshly thawed SMP, the DSC trace between 20 and 70 °C was essentially flat, but broad endotherms or exotherms appeared in this range with time, particularly between 40 and 50 °C. Their origin was not identified, but they are considered to be artifactual, considering their time-dependent development. By contrast, the sharp transition attributed to membrane-bound F_1ATPase was stable with time and highly reproducible.

Enzyme Inhibition. The effects of dibucaine and tetracaine on the ATPase activity of F_1ATPase and SMP are compared in Figure 4. These measurements were carried out in the same sucrose buffer employed in the DSC work, supplemented with the additional components required for the enzyme assay (see Materials and Methods). The results obtained with F_1ATPase in this buffer are essentially identical with those previously reported (Chazotte et al., 1982); viz., the anesthetics give partial inhibition as evidenced by the nonzero asymptotic behavior of the inhibition curves as the anesthetic concentration is raised. The lower asymptote for dibucaine as compared to

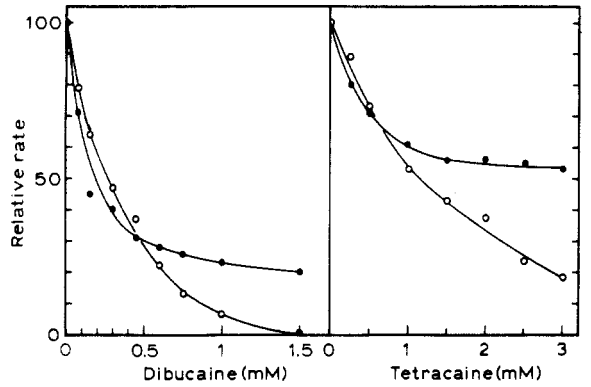

FIGURE 4: ATPase activity of F_1ATPase and SMP as a function of the dibucaine and tetracaine concentrations, expressed as the percent of the activity in the absence of added inhibitor: (●) F_1ATPase; (○) SMP. The assays were carried out at 25 °C under the conditions described in the text.

tetracaine is also in agreement with our previous findings. Different results were given by SMP in sucrose buffer, however. In our earlier work, partial inhibition was observed with SMP as well as with F_1ATPase, but it can be seen in Figure 4 that the SMP ATPase activity tends toward zero as the anesthetic concentration is raised. This appears to be evidence of a buffer effect in SMP which deserves further attention.

Two points are worthy of special note in regard to Figures 3 and 4. First, the concentration ranges of dibucaine and tetracaine which cause appreciable depression of T_m are the same as those which give enzyme inhibition. This is evidence that the molecular perturbations for both of these phenomena are probably closely related. Second, the finding that higher concentrations of anesthetics abolish the endothermic transition of SMP but not of F_1ATPase has its parallel in the observed complete inhibition of SMP by anesthetics as compared to partial inhibition of F_1ATPase.

Effective Anesthetic Concentration. It may be expected that in the SMP suspensions a certain fraction of anesthetic will be absorbed by the membrane lipids, thereby decreasing the free solution concentration. This effect will be greater in solutions prepared for DSC measurements than for enzyme assays on account of the considerably higher membrane concentrations employed in the former case. We assessed the magnitude of this effect for dibucaine. SMP suspensions at the same protein concentration as used for DSC measurements (2.3 mg/mL) were equilibrated with dibucaine, after which the SMP were pelleted by centrifugation and the dibucaine was determined spectrophotometrically in the supernatant. Table II gives the results obtained. It can readily be seen that the supernatant concentration was considerably lower than the initial solution average concentration; the difference must be due to dibucaine which was absorbed, adsorbed, or occluded by the pelleted SMP.

These results raise an interesting question. In evaluating the sensitivity of the DSC peak of SMP to anesthetics, should one use the solution average anesthetic concentration (as was done in Table I and in Figures 3 and 4), or the free solution concentration (middle column of Table II), or the elevated concentration present in the membranous pellet? The F_1ATPase and SMP transition temperatures are lowered by about the same amounts by the same total dibucaine concentrations (Figure 4), but if the data in Figure 4 were replotted using the supernatant concentrations of dibucaine given in Table II for SMP, the membrane-bound F_1ATPase would appear to be considerably more sensitive than the free F_1ATPase to anesthetic effects. Alternatively, the effective

Table II: Concentration Dependence of Dibucaine Uptake by SMP

dibucaine concn[a] (mM)		
total added	SMP supernatant	loss due to SMP binding
0.15	0.027	0.123
0.30	0.10	0.20
0.62	0.30	0.32
1.05	0.60	0.45
1.53	1.04	0.49

[a] Dibucaine concentrations were determined spectrophotometrically by using ϵ_{mM} = 4.40 at 326 nm (Vanderkooi, 1984). The SMP protein concentration was 2.3 mg/mL.

dibucaine concentration may be greater than the solution average concentration on account of the concentrating effect of absorption by the membrane lipids.

Discussion

Differential scanning calorimetry has proved to be a remarkably sensitive tool for studying the interactions of F_1ATPase with local anesthetics. One might expect that this method will also prove useful for investigating the interactions of other inhibitors, as well as the normal substrates, with this enzyme. Preliminary studies have already indicated that the calorimetric results are sensitive to buffer composition.

The fraction of total SMP protein which is F_1ATPase can be computed from the ratio of the enthalpy values given in Table I by making the assumption that the molar calorimetric enthalpy of thermal denaturation is the same for membrane-bound and extracted F_1ATPase. Since ΔH_d is 5 cal/g for SMP and 19 cal/g for F_1ATPase, roughly 25% of SMP protein appears to be F_1ATPase. This is a lower limiting value, considering that the extracted enzyme is probably less than 100% pure.

The calorimetric enthalpy of denaturation of F_1ATPase is considerably larger on a weight protein basis, by a factor of 3 or more, than values previously reported for monomeric globular proteins but is of comparable magnitude to that found for collagen (Privalov, 1982). These uncommonly large ΔH_d values for F_1ATPase and collagen may be related to the complex quaternary structures of the proteins.

The van't Hoff molar enthalpies (ΔH_{vh}) were computed by using eq 1 and are included in Table I. Since ΔH_{vh}, as calculated, is directly proportional to peak height (C_{max}) and inversely proportional to peak area (ΔH_d), the large ΔH_{vh} values may be seen either as a reflection of or as the cause of the remarkably sharp DSC peaks given by F_1ATPase. A comparison of the ΔH_{vh} values given in Table I for F_1ATPase and SMP shows that they are nearly the same; this is further evidence that membrane-bound F_1ATPase is responsible for the observed SMP peak.

The molar calorimetric enthalpies, ΔH_{cal}, may be calculated by multiplying ΔH_d by the molecular weight of F_1ATPase, taken as 360 000 (Senior, 1979). The values so obtained are included in Table I; these are underestimates of the true values by an amount in proportion to the degree of impurity in the F_1ATPase (probably not more than 10%; Chazotte et al., 1982). No ΔH_{cal} values are shown for SMP since it would be meaningless to calculate them for such a heterogeneous system. The $\Delta H_{cal}/\Delta H_{vh}$ ratios remain farily constant for all anesthetic concentrations since the van't Hoff enthalpies decrease in a parallel manner to the decrease in calorimetric enthalpies. The fact that this ratio is much greater than unity (i.e., 13–20) indicates that the protein denaturational transition responsible for the endotherm does not occur in a single cooperative step. By contrast, for most simple globular proteins,

the $\Delta H_{cal}/\Delta H_{vh}$ ratio is nearly unity, and this has been used as evidence that a two-state native to denatured equilibrium model was applicable (Privalov, 1979; Fukada et al., 1983). The large $\Delta H_{cal}/\Delta H_{vh}$ ratio found here may mean that there are many intermediate states in the thermal denaturation process (Krishnan & Brandts, 1978) or perhaps that different parts of the enzyme complex denature more or less independently to each other, so that the effective size of the cooperative unit responsible for the observed ΔH_{vh} is considerably smaller than the entire 360 000-dalton multimer.

F_1ATPase is probably rather unusual in regard to the sensitivity of its denaturational phase transition to anesthetics. We found that T_m for ribonuclease denaturation was unaffected by 1.5 mM dibucaine; Snow et al. (1978) reported that 10 mM tetracaine had no effect on the denaturation of spectrin, and Brandts et al. (1978) found no effect of chlorpromazine on the transitions of either spectrin or serum albumin.

The thermodynamic mechanism by which T_m is lowered by anesthetics cannot be established on the basis of the data now in hand. T_m would be expected to decrease if the anesthetics bound only, or preferentially, to the denatured form of the enzyme, thus shifting the (multistep) native $\rightleftharpoons$ denatured equilibrium to the right by a mass-action effect. In the case of the arabinose binding protein, the *increase* in T_m which occurred upon the addition of L-arabinose or D-galactose was quantitatively interpreted on the assumption that the ligands bind only to the *native* conformation (Fukada et al., 1983). The F_1ATPase reaction with anesthetics is more complex than this, however, since the enzyme inhibition studies, which were carried out at temperatures far below that at which denaturation begins, clearly demonstrate that the anesthetics do interact with the native state. We have, in addition, recently found that the local anesthetics protect F_1ATPase from cold inactivation at 0 °C. The same range of concentrations which give enzyme inhibition and lowering of the T_m also give this low-temperature protection, which suggests that these three effects may have a common molecular mechanism. [Palatini (1982) has previously noted that the tricyclic antipsychotics also protect this enzyme from low-temperature inactivation.] The T_m lowering may therefore be due in whole or in part to the conformational perturbation effects caused by anesthetics on the native state, although a mass-action effect resulting from a preferential binding to the denatured state cannot be ruled out as a contributory mechanism. How, in molecular terms, the anesthetics can both stabilize the enzyme at low temperature and destabilize it at high temperature is an intriguing question; perhaps the answer is related to the temperature dependence of the hydrophobic forces which are involved in the conformational stability of this protein.

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

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