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Inhibition by Trifluoperazine of ATP Synthesis and Hydrolysis by Particulate and Soluble Mitochondrial F_1: Competition with $H_2PO_4^-$

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The effect of trifluoperazine (TFP) on the ATPase activity of soluble and particulate F_1-ATPase and on ATP synthesis driven by succinate oxidation in submitochondrial particles from bovine heart was studied at pH 7.4 and 8.8. At the two pH, TFP inhibited ATP hydrolysis. Inorganic phosphate protected against the inhibiting action of TFP. The results on the effect of various concentrations of phosphate in the reversal of the action of TFP on hydrolysis at pH 7.4 and 8.8 showed that $H_2PO_4^-$ is the species that competes with TFP. The effect of TFP on oxidative phosphorylation was studied at concentrations that do not produce uncoupling or affect the aerobic oxidation of succinate ($<15\,\mu$M). TFP inhibited oxidative phosphorylation to a higher extent at pH 8.8 than at pH 7.4; this was through a diminution in the V_{max}, and an increase in the K_m for phosphate. Data on phosphate uptake during oxidative phosphorylation at several pH showed that $H_2PO_4^-$ is the true substrate for oxidative phosphorylation. Thus, in both synthesis and hydrolysis of ATP, TFP and $H_2PO_4^-$ interact with a common site. However, there is a difference in the sensitivity to TFP of ATP synthesis and hydrolysis; this is more noticeable at pH 8.8, i.e., ATPase activity of soluble F_1 remains at about 40% of the activity of the control in a concentration range of TFP of 40–100 μM, whereas in oxidative phosphorylation 14 μM TFP produces a 60% inhibition of phosphate uptake.

KEY WORDS: Mitochondrial ATP synthase; trifluoperazine; oxidative phosphorylation; $H_2PO_4^-$; soluble F_1.

INTRODUCTION

The F_0F_1 complex of the inner mitochondrial membrane catalyzes the synthesis of ATP[2] using the energy of electrochemical H^+ gradients. The enzyme is formed by a membrane portion referred to as F_0 and a soluble multisubunit portion known as F_1 (for reviews see Walker *et al.*, 1982; Martins *et al.*, 1988; Futai *et al.*, 1989; Capaldi *et al.*, 1992; Pedersen and Amzel, 1993). The most obvious catalytic property of soluble F_1 is to hydrolyze ATP (Pullman *et al.*, 1960). The F_1 portion of the complex has six adenine nucleotide binding sites. Three of these are considered catalytic; the other three sites exert regulatory functions (Cross and Nalin, 1982; Weber *et al.*, 1985; Martins *et al.*, 1992; Jault and Allison, 1993). Several lines of evidence indicate that the enzyme has a high degree of cooperativity (Hackney and Boyer, 1978; Grubmeyer and Penefsky, 1981; Penefsky, 1988), and there are reports showing that the three catalytic sites may be heterogeneous (Bullough *et al.*, 1987; Fromme and Gräber, 1989).

Inhibitors such as oligomycin (McLennan and Tzagoloff, 1968), dicyclohexyl carbodiimide

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[2] Abbreviations: ADP, adenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate; AMP-PNP, adenylyl imidodiphosphate; DCCD dicyclohexylcarbodiimide; IMP-PNP, 5′-(β,γ-imido)triphosphate; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; TFP, trifluoperazine; Tris, tris(hydroxymethyl)aminomethane.

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(Hermolin and Fillingame, 1989), aurovertin (Cross and Nalin 1982; Vázquez-Laslop *et al.*, 1989), and nitrobenzofurazan (Ferguson *et al.*, 1976; Cross and Nalin, 1982) have been extensively used to ascertain important features of the membrane and soluble portions of the ATPase complex. In addition, there is a class of inhibitors whose action has been studied in relation to their molecular structure. These are relatively hydrophobic molecules that affect the function of F_1, i.e., fluphenazine (Palatini, 1982), chlorpromazine (Laikind *et al.*, 1982; Palatini, 1982), quinacrine (Laikind and Allison, 1983; Bullough *et al.*, 1989; Jault and Allison, 1993), trifluoperazine (TFP) (de Meis *et al.*, 1988; Dabbeni-Sala and Palatini, 1990; Dabbeni-Sala *et al.*, 1990), and local anesthetics such as lidocaine, dubicaine, and tetracaine (Vanderkooi *et al.*, 1981; Laikind *et al.*, 1982; Chazotte *et al.*, 1982; Kresheck *et al.*, 1985; Vanderkooi and Adade, 1986). Interestingly, for the case of TFP, there are data that indicate that its inhibitory action is reversed by phosphate (de Meis *et al.*, 1988). It has also been shown that some of these hydrophobic molecules interact with the F_0 portion of the mitochondrial complex (Dabbeni-Sala and Palatini, 1990; Dabbeni-Sala *et al.*, 1990). Regarding the characteristics of these inhibitors, it has been reported that their inhibitory potency decreases with increasing pH (Palatini, 1982), and that an increase in the hydrophobicity of the molecules favors its interaction with F_1 (Chazotte *et al.*, 1982).

This work further examines the inhibitory action of TFP on the hydrolytic activity of soluble and particulate F_1, and on oxidative phosphorylation by bovine heart submitochondrial particles. The results show that TFP inhibits synthesis and hydrolysis of ATP through a process that is affected by pH. The results also show that mono-ionized phosphate ($H_2PO_4^-$) competes with TFP for a common site. In addition, it is shown that at pH 8.8, TFP produces a partial inhibition of hydrolysis at concentrations that produce a higher inhibition of phosphate uptake driven by succinate oxidation.

MATERIALS AND METHODS

Nonradioactive chemicals were obtained from Sigma. [^{32}P] phosphate was obtained from ICN and purified as described elsewhere (de Meis, 1984). Bovine heart mitochondria were prepared according to Low and Vallin (1963). Submitochondrial particles and soluble F_1 were prepared as described elsewhere (Beltrán *et al.*, 1986). Submitochondrial particles were stored at −70°C and F_1 at 4°C as an ammonium sulfate suspension until the time of the experiment. Before each experiment, stored F_1 was centrifuged and resuspended in 20 mM Tris-acetate, 0.05 mM ATP, and 0.5 mM EDTA at pH 7.4. TFP dihydrochloride was used; it was dissolved in water before each experiment. Under UV light TFP binds covalently to proteins, including various subunits of the ATPase complex (Dabbeni-Sala and Palatini, 1990; Dabbeni-Sala *et al.*, 1990). This side reaction was avoided by carrying out all experiments in the dark.

ATPase Activity

ATPase activity was assayed spectrophotometrically with an ATP regenerating system by following the decrease in absorbance at 340 nm at 25°C. The standard reaction media at the indicated pH contained 50 mM Tris-acetate, 30 mM K-acetate, 3 mM phosphoenolpyruvate, 6 mM Mg-acetate, 8 units of pyruvate kinase, and 6 units of lactate dehydrogenase and the indicated ATP concentrations. When the ATPase activity of submitochondrial particles was assayed, the standard reaction media also contained 0.20 M sucrose and 1 μM rotenone. Variations in the standard mixture are detailed in the Results section. ATPase activity of soluble F_1 was assayed with 1 μg F_1/ml; submitochondrial particles were used at a protein concentration of 0.1 mg/ml.

Oxidative Phosphorylation

Submitochondrial particles (0.1 mg protein per ml) were incubated at 25°C in Erlenmeyer flasks under constant shaking in 0.4 or 0.65 ml of a mixture that contained 0.15 M sucrose, 50 mM Tris-acetate, 6 mM Mg acetate, 20 mM succinate, 2 mM ADP, [^{32}P]Pi (as indicated), 20 mM glucose, and 46 units/ml of hexokinase at the pH indicated. The reaction was stopped by mixing 0.25 or 0.5 ml of the reaction media with trichloroacetic acid to yield a final concentration of 5% in 1.5 ml. After formation of phosphomolybdate, [^{32}P]Pi was extracted five times with butyl acetate as described before (Beltrán *et al.*, 1986). Nonextractable [^{32}P]Pi was considered equivalent to the amount of ATP formed.

ATP-Dependent Transhydrogenase Reaction

This was measured as described by Ernster

and Lee (1967). The reaction mixture (1 ml) at the indicated pH contained 50 mM Tris-acetate, 6 mM Mg-acetate, 20 mM phosphate, 1 mM KCN, 57 mM ethanol, 17 μM NAD, 0.2 mM NADP, 83 μg alcohol dehydrogenase, 0.1 mg/ml of submitochondrial particles, and the concentrations of ATP indicated under Results. Temperature was 25°C.

Distribution of Bound and Free TFP in Submitochondrial Particles

TFP (50 μM) was incubated with submitochondrial particles in a final volume of 1 ml at room temperature. The mixture was centrifuged at 105,000 *g* for 40 min. To determine free TFP, the supernatants were withdrawn and their absorbance at 258 nm was measured. Bound TFP was calculated from the difference in total and free TFP.

Protein was determined according to Lowry *et al.* (1951) in the presence of 0.25% sodium deoxycholate using bovine serum albumin as standard.

Calculation of K_i for TFP in Soluble F_1

TFP proved to be a noncompetitive partial inhibitor of ATP hydrolysis by soluble F_1 (see Results). This inhibition was modeled assuming noncompetitive binding of TFP at a single class of sites with a common dissociation constant (K_i). In the following reaction sequence, the binding of substrate and TFP are considered independent; thus the complex TFP-enzyme-substrate retains a fraction (β) of the control activity.

$$\begin{array}{ccccc} E + S & \overset{K_s}{\rightleftharpoons} & ES & \xrightarrow{k_p} & E + P \\ + & & + & & \\ TFP & & TFP & & \\ K_i \Big\| & & K_i \Big\| & & \\ E^{TFP} + S & \overset{K_s}{\rightleftharpoons} & E^{TFP}S & \xrightarrow{\beta k_p} & E^{TFP} + P \end{array} \qquad (1)$$

In this case the velocity of reaction is

$$V = k_p[ES] + \beta k_p[E_{TFP}S] \qquad (2)$$

this may be expressed in relation to the V_{max} after substituting the free enzyme concentration as follows:

$$V = \frac{[S](1 + \beta[TFP]/K_i)V_{max}}{K_s(1 + [TFP]/K_i) + [S](1 + [TFP]/K_i)} \qquad (3)$$

This equation was used for a nonlinear regression analysis (Leatherbarrow, 1987) of the experimental data obtained from the effect of various concentrations of TFP on ATP hydrolysis by soluble F_1. Excellent fittings were obtained between experimental points and theoretical curves. From the equation, K_i and the residual activity factor (β) were obtained. The K_m for Mg^{2+}-ATP used was 0.2 mM (as determined from experiments in this work); the V_{max} varied between 50 and 60 μmol/min/mg in different experiments.

RESULTS

Effect of TFP on ATP Hydrolysis by Particulate and Soluble F_1

It has been described that the inhibition of ATPase activity of soluble F_1 by tricyclic molecules such as chlorpromazine, and by local anesthetics, i.e., fluphenazine and flupenthixol, decreases with increasing pH. From the changes of $CI_{50\%}$ (concentration required for half inhibition) at different pH, the lower inhibition of F_1 at alkaline pH was ascribed to the changes in the protonation state of the inhibitors (Palatini, 1982). To determine if the inhibition by TFP of ATP hydrolysis follows this pattern, its effect on the ATPase activity of submitochondrial particles and soluble F_1 was assayed at pH 7.4 and 8.8. In particles, the inhibition curves were similar at the two pH (Figs. 1A and B). In accordance with previous data (de Meis *et al.*, 1988), Fig. 1 also shows that at pH 7.4, 40 mM inorganic phosphate (Pi) protected against the inhibiting action of TFP, but it was now found that at pH 8.8, protection by this concentration of Pi was much lower.

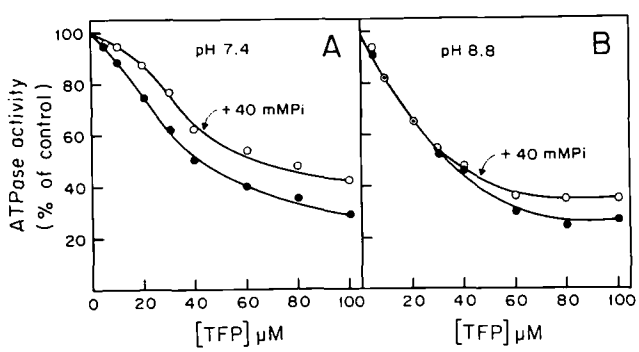

Fig. 1. Inhibition of ATPase activity of submitochondrial particles by TFP at pH 7.4 and 8.8. Protection by Pi. (A) ATPase activity was measured at pH 7.4 with increasing concentrations of TFP, in the presence (○, 100% = 0.165 μmol/min mg) and in the absence (●, 100% = 0.21 μmol/min mg) of 40 mM Pi. (B) ATPase activity measured at pH 8.8, with (○, 100% = 0.337 μmol/min mg) and without (●, 0.244 μmol/min mg) 40 mM Pi.

Table I. Inhibition Constants for TFP as Inhibitor of ATP Hydrolysis by Soluble F_1[a]

Activity	pH	K_i (μM)	Residual activity factor (β)
F_1-ATPase	7.4	15	0.2
F_1-ATPase + Pi 40 mM	7.4	50	0.4
F_1-ATPase	8.8	11	0.3
F_1-ATPase + Pi 40 mM	8.8	11	0.5

[a] Experimental data from Fig. 2 were fitted by nonlinear regression analysis in the model of noncompetitive partial inhibition described under Materials and Methods.

TFP also inhibited ATP hydrolysis by soluble F_1 (Figs. 2A and B). The sensitivity of the inhibiting effect of TFP to Pi was also lower at pH 8.8. From the kinetic model described under Materials and Methods, the K_i for TFP in F_1 was calculated (Table I). The values at the two pH were almost the same (15 and 11 μM, respectively). This indicates that regardless of the protonation state of TFP ($pK_{a_1} = 3.9$, $pK_{a_2} = 8.1$), the affinity of the enzyme for TFP did not vary in this pH range. On the other hand, at pH 7.4, 40 mM Pi increased the K_i for TFP to 50 μM.

In the data of Figs. 1 and 2 there are some additional features. The inhibiting effect of TFP on hydrolysis was partial; this was more noticeable at pH 8.8 with the soluble enzyme. In the range of 60–100 μM TFP, activity remained at about 40% of the control (Fig. 2B, Table I). Figures 1 and 2 also show that protection against TFP action by Pi was higher in the soluble than in the particulate enzyme.

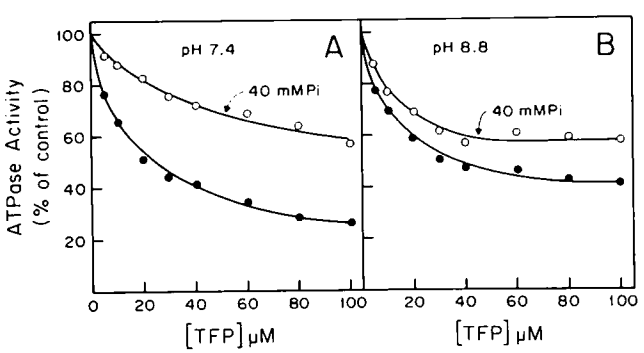

Fig. 2. Inhibition of ATPase activity of soluble F_1 by TFP at pH 7.4 and pH 8.8. Protection with Pi. (A) ATPase was assayed at pH 7.4 and the indicated concentrations of TFP, with (○, 100% = 34 μmol/min mg) and without (●, 100% = 48 μmol/min mg) 40 mM Pi. (B) ATPase activity was assayed at pH 8.8 in the presence (○, 100% = 38 μmol/min mg) and in the absence (●, 100% = 51 μmol/min mg) of 40 mM Pi.

Another finding was that at pH 7.4, the inhibition by increasing concentrations of TFP in soluble F_1 was hyperbolic, whereas in the particulate enzyme, the titration curve was sigmoidal.

Protection of TFP-Induced Inhibition of Hydrolysis by $H_2PO_4^-$

To account for the lower protective effect of Pi at pH 8.8 than at pH 7.4 (Figs. 1 and 2), it was thought that this could be due to the different concentrations of $H_2PO_4^-$ or HPO_4^{-2} that exist at the two pH values. To test this possibility, the effect of TFP on the ATPase activity of soluble F_1 incubated with various Pi concentrations was determined at pH 7.0 and 8.0 (Fig. 3). The results were plotted as function of overall Pi or $H_2PO_4^-$ concentrations. Lower Pi concentrations were required to prevent the action of TFP at pH 7.0 than at pH 8.0 (inset Fig. 3). On the other hand, when the inhibiting action of TFP was plotted as function of the concentration of $H_2PO_4^-$, there was a marked overlapping of the curves obtained at pH 7.0 and 8.0 (Fig. 3). Accordingly, it may be concluded that $H_2PO_4^-$ is the active, or the more active, species in the displacement of TFP from its inhibitory site. The data also indicate that the inhibition of TFP action by Pi is not due to a lowering of its effective

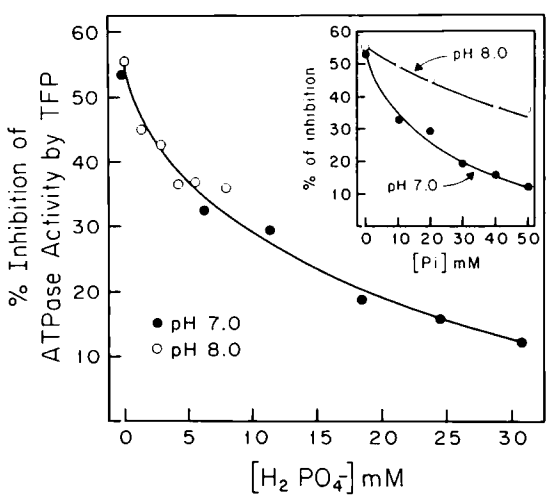

Fig. 3. Reversal of the inhibition of F_1 ATPase activity by TFP with Pi at pH 7.0 and 8.0. F_1-ATPase activity was measured in the presence of 25 μM of TFP at pH 7.0 (●, 100% without TFP = 53 μmol/min mg) or at pH 8.0 (○, 100% = 59 μmol/min mg) with increasing concentrations of Pi. The plot depicts the percent of inhibition of ATPase activity by 25 μM TFP against the concentration of $H_2PO_4^-$ at both pH. Inset: percent inhibition plotted against the total Pi concentration.

concentration in the media as a consequence of electrostatic interactions between positively charged TFP and negatively charged Pi, i.e., in such case it would be expected that HPO_4^{-2} would be more effective.

Molecules similar to TFP exert a noncompetitive inhibition of ATP hydrolysis (Palatini, 1982; Laikind *et al.*, 1982). Likewise, Lineweaver–Burke plots obtained from the activity of soluble F_1 incubated with TFP, at pH 7.4 and 8.8 and various Mg^{2+}-ATP concentrations, showed that the main effect of TFP was on the V_{max} (Table II). Only a small modification of the K_m for Mg^{2+}-ATP was observed. The noncompetitive type of inhibition of ATP hydrolysis by TFP is indicative that TFP does not perturb, at least to a large extent, the affinity of the enzyme for ATP, albeit $H_2PO_4^-$ protects against the inhibition (Fig. 3).

Effect of TFP on Electron Transport and the Transhydrogenase Reaction, and Its Distribution between the Particulate and Water Spaces in Submitochondrial Particles

Prior to studies on the effect of TFP on oxidative phosphorylation of submitochondrial particles, it was first necessary to determine its effect on the respiration and electrochemical H^+ gradients of such preparations. Also, the distribution of TFP between submitochondrial particles and the external water space was determined. TFP produced a marked inhibition of the aerobic oxidation of NADH (25 μM inhibited by about 50%). The oxidation of succinate was also inhibited, but to a much lower extent, i.e., 25 μM TFP diminished oxygen uptake by about 10% (not shown).

Attempts were made to determine the effect of TFP on the electrical potential with oxonol VI. No reliable measurements were obtained with this methodology due to complex formation between TFP and oxonol. To circumvent the problem, the effect of TFP on the ratio of ATP hydrolyzed per NADP reduced in the ATP-supported transhydrogenase reaction (Ernster and Lee, 1967) was determined. The rationale was that a lack of effect of TFP (or any other agent) in the ratio of ATP hydrolyzed per NADPH formed would be indicative that energy is not dissipated. On the other hand, energy leaks would produce a lower availability of H^+ gradients for reduction of NADP, and in consequence, an increase in the ratio of ATP hydrolyzed per NADP reduced. The reliability of the method is shown in Fig. 4A. When the transhydrogenase reaction and ATP hydrolysis were measured under identical conditions at various rates of ATP hydrolysis as controlled by substrate concentration, the ratio of ATP hydrolyzed per NADP reduced was constant. This indicated that the rate of the transhydrogenase reaction was governed by the rate of ATP hydrolysis. The effect of increasing concentrations of TFP on the energy-dependent transhydrogenase reaction supported by ATP hydrolysis at pH 7.4 and 8.8 is shown in Figs. 4B and C. The ratio of NADPH formed per ATP hydrolyzed was higher at pH 8.8 than at pH 7.4. However, at the two pH TFP decreased the ATP-driven reduction of NADP (Fig. 4A). Up to 15 μM TFP, this was mostly a consequence of inhibition of ATP hydrolysis, since there was a nearly constant ratio of ATP hydrolyzed per NADPH formed. At TFP concentrations higher than 15 μM, the ratio increased. Thus, depending on its concentration, TFP exerts two effects: (i) at concentrations lower than 15 μM, TFP inhibits ATP hydrolysis, and in consequence there is a diminution in NADPH formation; (ii) at TFP concentrations higher than 15 μM, TFP also exerts an uncoupling effect; this was evidenced by the increase in the ratio of ATP hydrolyzed/NADP reduced. It is noted that these two effects of TFP are reversible by dilution (not shown).

The partition of several anesthetic drugs into lipid bilayers increases with increasing pH (Miyasaki *et al.*, 1992); this has been attributed to an enrichment of the membranous space with the nonprotonated molecules. To explore if the effects of TFP on the ATPase complex could be related to its concentration in the membrane or in the water space of submitochondrial particles, the distribution of a fixed concentration of TFP was determined at pH 7.4 and 8.8

Table II. Kinetic Constants for Soluble F_1-ATPase with Different Concentrations of TFP at pH 7.4 and 8.8[a]

pH	[TFP] (μM)	K_m for ATP (mM)	V_{max} (μmol/min mg)
7.4	0	0.2	52
7.4	5	0.2	41
7.4	20	0.1	25
7.4	40	0.1	19
7.4	50	0.1	18
8.8	0	0.2	56
8.8	5	0.2	45
8.8	20	0.1	30
8.8	40	0.2	26

[a] ATPase activities were measured as described under Materials and Methods at the indicated pH and TFP concentrations, and Mg^{2+}-ATP concentrations that ranged between 0.1 and 1.0 mM. K_m and V_{max} were calculated from Lineweaver–Burk plots.

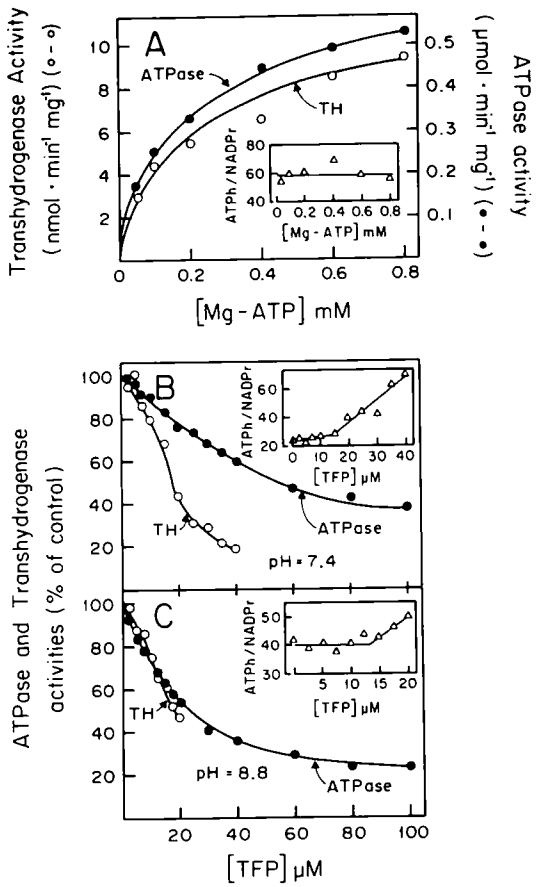

Fig. 4. Degree of coupling between ATPase and ATP-dependent transhydrogenase activities in submitochondrial particles at various ATP and TFP concentrations. (A) ATPase and ATP-dependent transhydrogenase (TH) activities were measured as described in Materials and Methods, at different Mg^{2+}-ATP concentrations, at pH 8.8. (B) ATPase (●, 100% = 0.21 μmol/min mg), and transhydrogenase (○, 100% = 10.2 nmol/min mg) activities were measured at pH 7.4, with 3 mM Mg^{2+}-ATP at the indicated TFP concentrations. (C) ATPase (●, 100% = 0.373 μmol/min mg) and transhydrogenase (○, 100% = 8.8 nmol/min mg) were measured at pH 8.8 with increasing TFP concentrations. All activities were measured in the presence of 20 mM Pi. The ATP-independent transhydrogenase activities (with FCCP 1 μM) were substracted from the total activities. Thus the reported transhydrogenase activities are ATP dependent.

at several concentrations of particles (Fig. 5). It was found that at the two pH, the differences in the distribution of TFP in the two spaces were not statistically significant (Figs. 5A and B). Note that as particle concentration was increased, there was a dilution of TFP in the particulate space, and in consequence, TFP concentration in the two spaces decreased. This is of importance in studies on the inhibition of ATP synthesis by TFP. In fact, the inhibition of oxidative

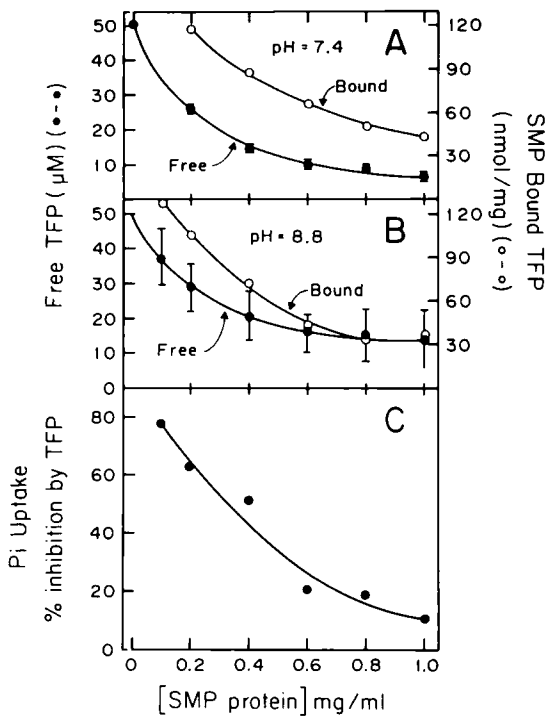

Fig. 5. Effect of the concentration of submitochondrial particles (SMP) on the distribution of TFP and on the inhibition of oxidative phosphorylation. (A) Free (●) and SMP bound (○) TFP at pH 7.4 with increasing concentrations of SMP. (B) Same as in A, but the pH was 8.8. Standard deviations are shown only for the measured parameter (free TFP) in four (A) and three (B) separate determinations. (C) Oxidative phosphorylation was measured in the presence of 40 μM TFP with increasing concentrations of SMP at pH 7.4. 100% of activity (without TFP) was constant (87 nmol/min mg) at the different submitochondrial particle concentrations.

phosphorylation by 40 μM TFP diminished as the concentration of the particles increases (Fig. 5C).

Effect of TFP on Oxidative Phosphorylation

The results described in the previous section were used for standardizing the conditions for studying the effect of TFP on oxidative phosphorylation. This was determined with succinate as substrate at a concentration of particles of 100 μg/ml. In addition, to avoid an uncoupling action of TFP, the experiments on oxidative phosphorylation were carried out at TFP concentrations lower than 15 μM.

Figure 6 shows the effect of TFP on oxidative phosphorylation by submitochondrial particles at pH 7.4 with 1.25 and 20 mM Pi, and at pH 8.8 with 20 mM Pi. At pH 7.4, inhibition of oxidative phosphorylation by TFP was higher with 1.25 mM than with 20 mM Pi. At pH 8.8 and 20 mM Pi, TFP

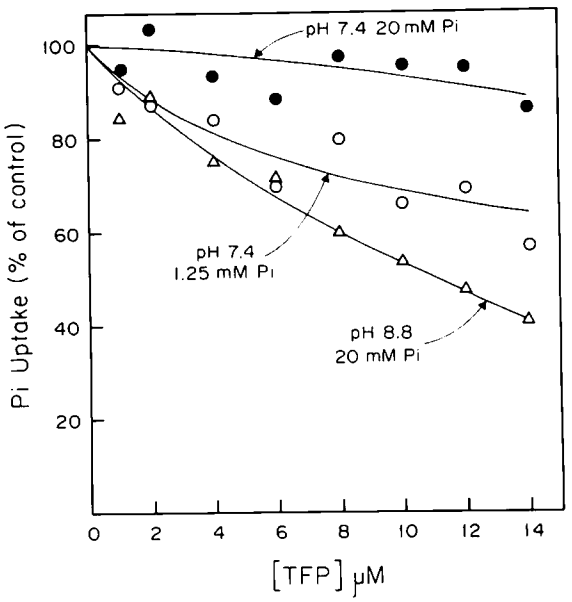

Fig. 6. Inhibition of oxidative phosphorylation by TFP at pH 7.4 and pH 8.8. Oxidative phosphorylation was measured in submitochondrial particles as described under Materials and Methods in the following conditions: pH 7.4 and 20 mM Pi (●, 100% = 125 nmol/min mg); pH 7.4 and 1.25 mM Pi (○, 88 nmol/min mg); pH 8.8 and 20 mM Pi (△), 100% = 81 nmol/min mg).

inhibited oxidative phosphorylation more effectively than at pH 7.4. In experiments not shown it was observed that at pH 8.8, the percent inhibition of oxidative phosphorylation by various concentrations of TFP was markedly similar with 1 and 20 mM Pi in the reaction media. In this respect, it is pointed out

Table III. Effect of TFP on the Kinetic Constants for Pi and $H_2PO_4^-$ in Oxidative Phosphorylation at Different pH[a]

pH	[TFP] (μM)	K_m for Pi (mM)	K_m for $H_2PO_4^-$ (mM)	V_{max} (nmol/min mg)
7.4	0	0.5	0.2	116
7.4	14	1.2	0.5	103
8.8	0	4.4	0.1	67
8.8	14	9.6	0.2	35

[a] Oxidative phosphorylation was assayed as described under Materials and Methods at the indicated pH in a Pi concentration range that varied between 0.5 and 20 mM at pH 7.4, and between 2 and 40 mM at pH 8.8. K_m and V_{max} values were calculated from Linewaver–Burk plots.

that at pH 7.4, 1.25 and 20 mM total Pi have 0.49 and 7.85 mM $H_2PO_4^-$ respectively, whereas at pH 8.8, the respective $H_2PO_4^-$ concentrations are 0.02 and 0.49 mM. Thus, it is possible that the absence of an effect of $H_2PO_4^-$ on the inhibition of oxidative phosphorylation produced by TFP at pH 8.8 is due to the relatively low concentrations of $H_2PO_4^-$ that were achieved at this pH.

An additional point in the data in Fig. 6 is that with 0.49 mM $H_2PO_4^-$ (with 1.25 mM total Pi at pH 7.4, and 20 mM total Pi at pH 8.8), inhibition was higher at pH 8.8. Thus at pH 8.8, irrespectively of the overall concentration of Pi or $H_2PO_4^-$, TFP inhibited oxidative phosphorylation more effectively than at pH 7.4.

Studies of the kinetics of the effect of TFP on oxidative phosphorylation at various concentrations

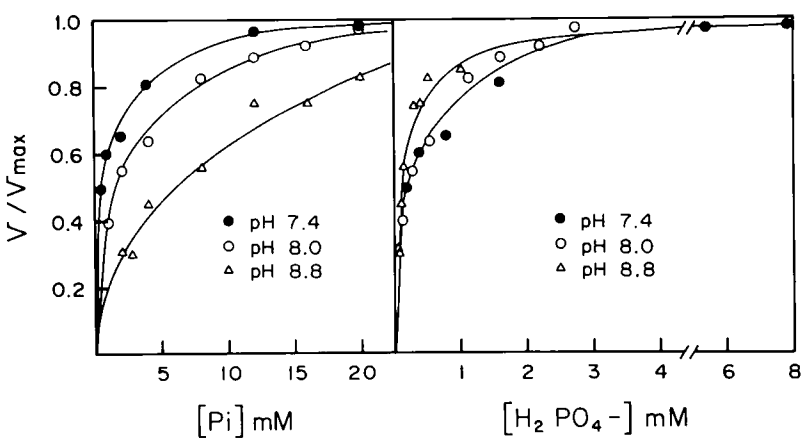

Fig. 7. Oxidative phosphorylation at different pH and Pi concentrations. Oxidative phosphorylation was measured in submitochondrial particles as described under Materials and Methods at the Pi concentration and pH shown. The ratio of phosphorylation velocity (V) over V_{max} (V/V_{max}) was plotted vs. total Pi (left) or against the concentration of $H_2PO_4^-$ (right). (●) pH 7.4; (○) pH 8.0; (△) pH 8.8. For the values of K_m see text.

of Pi showed that TFP exerted a mixed type of inhibition. At pH 8.8, the main modification was on the V_{max}; at pH 7.4, the change in V_{max} was much lower. However, at pH 7.4 and 8.8, TFP increased the K_m for Pi (or $H_2PO_4^-$) by a factor of 2 (Table III). Hence, a common feature of TFP at the two pH is that TFP competes with $H_2PO_4^-$ in particulate F_1.

$H_2PO_4^-$ as Substrate for Oxidative Phosphorylation

In view of the relation between TFP and $H_2PO_4^-$, it was of interest to explore if this species is the true substrate for oxidative phosphorylation. In the experiment of Fig. 7, Pi uptake in oxidative phosphorylation was measured at pH 7.4, 8.0, and 8.8 and various Pi concentrations. When Pi uptake was plotted as function of the overall concentration of Pi, the curves at the three pH were markedly different (Fig. 7A). From Lineweaver–Burk plots, the calculated K_m for total Pi at pH 7.4, 8.0, and 8.8 were 0.6, 1.5, and 4.9 mM respectively. In contrast, when Pi uptake at pH 7.4 and 8.0 was plotted against $H_2PO_4^-$ concentration, there was an overlapping of the points of the curves at pH 7.4 and 8.0 (Fig. 7B). The points obtained at pH 8.8 fell on a different curve. However at pH 7.4, 8.0, and 8.8, the K_m for $H_2PO_4^-$ were in the same range, i.e., 0.2, 0.2, and 0.1 mM respectively. These results are strongly suggestive that $H_2PO_4^-$ is the species of preference in oxidative phosphorylation.

DISCUSSION

Effect of pH on the Inhibitory Action of TFP

The studies on the effect of TFP on ATP synthesis and hydrolysis at various pH show that pH affects the action of TFP. For instance, inhibition of ATP synthesis in submitochondrial particles by TFP is lower at pH 7.4 than at pH 8.8. Furthermore, at pH 7.4 inhibition of synthesis and hydrolysis in submitochondrial particles run in parallel, whereas at pH 8.8 synthesis is more sensitive than hydrolysis. Also, it is noted that an increase in pH produces a higher inhibition of ATP synthesis in submitochondrial particles, but a lower inhibition of soluble F_1. Since at the two pH the K_i of TFP for soluble F_1 is nearly the same, the lower inhibition by TFP of F_1 at pH 8.8 would not seem to be due to differences in the protonation state of TFP; rather the differences in inhibition would be due to changes in the protonation of the enzyme. Also it was found that partition factors cannot account for the different sensitivity to TFP, since in submitochondrial particles the concentrations of TFP in the particles and the external water space did not vary with pH. In consequence, it would appear that the F_0F_1 complex has different characteristics at the two pH, and that these affect the response of the enzyme to TFP.

Relation between TFP and $H_2PO_4^-$ in Hydrolysis and Synthesis of ATP

Although there are differences in the action of TFP on the F_0F_1 complex at pH 7.4 and 8.8, the interplay between Pi and TFP is maintained at the two pH: (i) at the two pH, equal concentrations of $H_2PO_4^-$ produce almost identical protection against TFP action in ATP hydrolysis by soluble F_1, and (ii) at the two pH, TFP increases by about twofold the K_m for $H_2PO_4^-$ in oxidative phosphorylation. Taken together these findings strongly suggest that TFP and $H_2PO_4^-$ interact with the same locus of the F_1 portion. From measurements of Pi binding to soluble F_1 from bovine mitochondria (Kasahara and Penefsky, 1978) and *E. coli* (Al-Shawi and Senior, 1992), it has been proposed that $H_2PO_4^-$ is the preferred substrate for oxidative phosphorylation. Further evidence for this proposal is now provided by the direct measurement of oxidative phosphorylation in submitochondrial particles at several pH (Fig. 7). Thus, these data suggest that TFP acts on the catalytic sites of soluble and particulate F_1, and that these sites are involved in both synthesis and hydrolysis of ATP. In this respect it is also noted that some amphipatic cations diminish derivatization by DCCD of the glutamate 199 which is at the catalytic site (Laikind *et al.*, 1982; Bullough *et al.*, 1985; Bullough *et al.*, 1989), and that quinacrine mustard, one of these amphipatic cations, also interferes with ADP binding at the catalytic site (Laikind and Allison, 1983). However, Kasahara and Penefsky (1978) showed that in F_1 there are two clearly distinguishable Pi binding sites, one of which does not appear to be catalytic. In consequence, it is possible that by binding to the latter site, TFP affects the catalytic properties of F_1. In this respect, it is worth noting that the recently described (Abrahams *et al.*, 1994) crystal structure of F_1 shows that in the C-terminal region of the β-subunit which is in close contact with the γ-subunit and far from the catalytic site, lies a region that may bind amphipatic cations.

Effect of TFP on ATP Synthesis and Hydrolysis

The site with which TFP interacts becomes of particular relevance if it is considered that the inhibition of hydrolysis by TFP is not total, the partial effect being more apparent in soluble F_1 at pH 8.8. Furthermore, at pH 8.8, equivalent concentrations of TFP produce a partial inhibition of ATPase activity and a higher inhibition of synthesis. It is possible that the inhibition of oxidative phosphorylation by TFP results from its interaction with sites other than those in F_1. Indeed, the sigmoidal type of inhibition as a function of TFP concentration of ATP hydrolysis and synthesis in submitochondrial particles at pH 7.4 (Figs. 1 and 6), the lower protection achieved by Pi in F_0F_1 (Figs. 1 and 6; Table III) than in soluble F_1 (Figs. 2 and 3), together with findings (Dabbeni-Sala and Palatini, 1990; Dabbeni-Sala *et al.*, 1990) that show that photomodified TFP covalently bound to the F_0 portion of a reconstituted system inhibits hydrolysis, argue in favor of this possibility. However, the questions that remain are why the inhibition of ATPase activity by TFP is partial, and why in submitochondrial particles a partial inhibition of ATP hydrolysis is accompanied by a higher inhibition of synthesis at pH 8.8.

A different sensitivity of phosphorylation and hydrolysis to increasing concentrations of various inhibitors has been described (Roberton *et al.*, 1968; Linnett and Beechey, 1979; Matsuno-Yagi and Hatefi, 1984; Van Der Bend *et al.*, 1985). However, with the exception of AMP-PNP and IMP-PNP (Penefsky, 1974; Schuster *et al.*, 1976) high concentrations of the inhibitors produce almost full inhibition of ATP synthesis and hydrolysis. This is not the case for TFP, i.e., at pH 8.8, inhibition of hydrolysis in soluble F_1 levels off at about 40% of the activity of the control. Thus it would appear that the interaction of TFP with the F_0F_1 complex results in an enzyme in which the events that lead to ATP synthesis are more seriously impaired than those involved in hydrolysis. Further studies on the mechanism of action of TFP should shed light on the interrelation between the catalytic sites of F_1.

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