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Thiols in Oxidative Phosphorylation: Inhibition and Energy-Potentiated Uncoupling by Monothiol and Dithiol Modifiers†

Takao Yagi and Youssef Hatefi*

ABSTRACT: Three apparently different modifications of submitochondrial particles (SMP) or ATP synthase preparations (complex V) inhibit oxidative phosphorylation and ATP–$^{32}P_i$ exchange activities, all of which are reversible by addition of mono- or dithiols. (a) Triphenyltin chloride inhibits ATP synthesis and hydrolysis without uncoupling. The inhibition by triphenyltin chloride is reversible by addition of β-mercaptoethanol, dithiothreitol, or dihydrolipoamide. (b) Factor B is a water-soluble protein of M_r (11–12) × 10^3, contains a vicinal dithiol, and is required for energy transfer to and from F_1-ATPase when tested with SMP-rendered factor B deficient by extraction with ammonia–ethylenediaminetetraacetic acid (EDTA) (AE-SMP). Treatment of factor B with mono- and dithiol modifiers, such as *p*-(chloromercuri)benzenesulfonate (PCMPS), Cd^{2+}, or diazenedicarboxylic acid bis(dimethylamide) (diamide), inhibits factor B. This inhibition is reversed by addition to modified factor B of appropriate mono- and dithiol compounds. Preparations of AE-SMP are partially F_1 deficient and partially uncoupled. The uncoupling can be repaired completely by addition of factor B or low levels of oligomycin, or to a large extent by addition of F_1-ATPase + oligomycin sensitivity conferring protein. (c) SMP, AE-SMP, and complex V can be completely uncoupled by treatment at 30 °C with phenylarsine oxide, Cd^{2+}, diamide, PCMPS, monobromobimane, and mono- and bifunctional maleimides. The uncoupling by these reagents is potentiated by membrane energization. Uncoupling by diamide is ≥80% reversed by dihydrolipoamide or β-mercaptoethanol, the former being much more potent. Dithiothreitol and dithioerythritol are poorly effective. The uncoupling by Cd^{2+} and PCMPS is also reversible, but only ≤50%. Unlike the partial uncoupling of factor B deficient particles, the energy-potentiated uncoupling by the above mono- and dithiol modifiers is not repaired by addition of oligomycin or dicyclohexylcarbodiimide. The target for triphenyltin chloride may or may not be a thiol. However, the phenomena described in (b) and (c) suggest the presence in the membrane sector (F_0) of the ATP synthase complex of possibly two sets of dithiols involved in energy transfer. One of these is factor B, which is probably located between F_1 and the inhibition site of oligomycin in F_0. The other putative dithiol causes complete and reversible uncoupling when modified. Since this uncoupling is not repairable by oligomycin, it appears phenomenologically that the lesion is on the cytosolic side of the oligomycin block point of the proton channel.

Mono- and dithiols have been implicated in the energy-linked functions of membranes [for example, see Sanadi et al. (1968), Abou-Khalil et al. (1975), Godinot et al. (1977, 1981), Conn et al. (1981), and Moroney et al. (1982)], and it has been suggested that dithiol–disulfide interchange might play a role in energy-transducing processes (Robillard & Konings, 1982). In mitochondria, mono- and dithiols appear to be involved in oxidative phosphorylation and maintenance of membrane integrity (Siliprandi et al., 1974; Abou-Khalil et al., 1975; Godinot et al., 1981; Le-Quoc & Le-Quoc, 1982). Among the mitochondrial components which contain thiols essential for oxidative phosphorylation is factor B, which was discovered by Sanadi and co-workers [for a review, see Sanadi (1982)] and purified by You & Hatefi (1976). Factor B is a water-soluble protein of M_r (11–12) × 10^3 according to You & Hatefi (1976) and 15 × 10^3 according to Sanadi (1982). It contains an essential vicinal dithiol (Stiggall et al., 1979a; Joshi & Hughes, 1981) and appears to be required for energy transfer to and from F_1-ATPase. In chloroplasts, it has been demonstrated that illumination unmasks thiol groups in the γ subunit which can be modified by maleimide derivatives, resulting in inhibition of photophosphorylation (McCarty & Fagan, 1973; Moroney et al., 1982). The bifunctional ma-

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leimide *o*-phenylenedimaleimide (OPDM)[1] was shown to be a potent inhibitor of photophosphorylation (Weiss & McCarty, 1977). The inhibition required illumination of the thylakoids and resulted in cross-linking within the γ subunit and uncoupling due to enhanced proton permeability of the thylakoid membranes. Addition of DCCD to OPDM-treated thylakoids restored proton uptake, which agreed with the interpretation that increased proton permeability was a consequence of cross-linking and structural modification of the γ subunit of the ATPase.

The present studies will show that there exists in mitochondria a locus phenomenologically similar to that which in chloroplast ATPase is modified by mono- and bifunctional maleimides. This locus, which is probably a dithiol, is different from the inhibition site of trialkyltin chloride or aryltin chloride. The modification of this locus by several monothiol and dithiol reagents occurs more rapidly under energized conditions and results in uncoupling and enhancement of ATPase activity. The effect of membrane energization can be prevented in the presence of uncouplers, and the uncoupling effect of thiol modifiers can be reversed by the addition of appropriate monothiol and dithiol compounds. Modification of factor B by appropriate thiol inhibitors and the inhibitory effect of triphenyltin chloride (TPT-Cl) can also be reversed by thiol compounds. However, TPT-Cl inhibits ATPase activity and does not cause uncoupling, and factor B removal to the extent that ATP-energized reactions of SMP are diminished by ~90% causes only small changes in the ATPase activity and the ability of the membranes to maintain a membrane potential.

Materials and Methods

Bovine heart mitochondrial SMP (Hansen & Smith, 1964), factor B and factor B deficient particles (AE-SMP) (You & Hatefi, 1976), ASU particles (SMP deficient in F_1 and OSCP) (Racker & Horstman, 1967), the ATP synthase complex (complex V) (Stiggall et al., 1979b), F_1-ATPase (Senior & Brooks, 1970), and OSCP (Senior, 1971) were prepared according to the references given. Oxidative phosphorylation, ATP-driven electron transfer from succinate to NAD, and transhydrogenation from NADH to the 3-acetylpyridine analogue of NADP (AcPyADP) (Hatefi et al., 1982) and ATP hydrolysis using the ATP-regenerating system and ATP–$^{32}P_i$ exchange (Stiggall et al., 1979b) were assayed essentially according to the references cited. Membrane potential changes were monitored by the absorbance change of oxonol VI at 630 minus 603 nm as described previously (Yagi et al., 1984), using an Aminco DW-2a dual-wavelength spectrophotometer. Any variations from these procedures and other details are described in the figure legends. Protein concentration was determined by the method of Lowry et al. (1951).

Sodium ascorbate, hexokinase, MalNEt, lactic dehydrogenase, pyruvate kinase, phosphoenolpyruvate, diamide, lipoamide, OPDM, and PCMPS were obtained from Sigma; ADP, NAD(H), and NADP were from P-L Biochemicals; ATP and oligomycin were from Boehringer; antimycin, dithiothreitol, and monobromobimane were from Calbiochem; TPT-Cl was from ICN Pharmaceuticals; DCCD was from K & K Laboratories; PAO was from Aldrich; $^{32}P_i$ was from ICN; TMPD was from Eastman-Kodak; rotenone was from S. B. Penick. Oxonol VI was a generous gift of Dr. W. G. Hanstein, University of Bochum. Other chemicals were reagent grade or of the highest quality available.

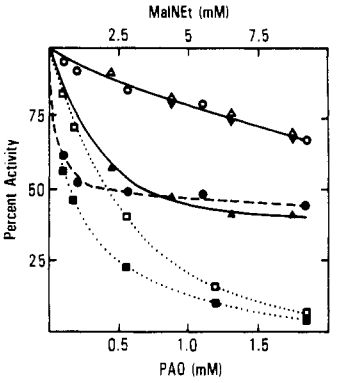

FIGURE 1: Energy-potentiated inhibition of ATP–$^{32}P_i$ exchange and oxidative phosphorylation, respectively, by PAO and MalNEt. SMP at 0.8–1.0 mg/mL in a mixture containing 0.25 M sucrose, 50 mM Tris–acetate (pH 7.5), and 1.0–1.25 mM EDTA was incubated at 30 °C for 30 min with PAO (added in dimethylformamide) (triangles) and for 10 min (circles) or 30 min (squares) with MalNEt at the final concentrations shown in the figure. Where indicated, 20 mM sodium succinate (▲, ●, ■) or 20 mM sodium succinate plus 540 μM 2,4-dinitrophenol (▼) was also present. After incubation, the tubes containing SMP were transferred to an ice bath, and the particles were assayed for activity. The assay for ATP–$^{32}P_i$ exchange was carried out at 30 °C in a mixture containing 0.25 M sucrose, 50 mM Tris–acetate (pH 7.5), 4.25 mM $MgCl_2$, 20 mM $K^{32}P_i$, 1 mM ATP, 1 μM antimycin A, and 91 μg/mL PAO-treated SMP. At zero PAO concentration, the exchange activities in the absence and presence of succinate in the preincubation mixture were, respectively, 146 and 144 nmol min^{-1} mg^{-1} (uncorrected for ATP hydrolysis during exchange), and the exchange activity of the particles treated with 2,4-dinitrophenol was 78% of the control. The assay mixture for oxidative phosphorylation at 30 °C contained 0.25 M sucrose, 25 mM Tris–acetate (pH 7.5), 25 mM glucose, 68.5 μg/mL hexokinase, 1 μM antimycin A, 4.25 mM $MgCl_2$, 1 mM ADP, 20 mM $K^{32}P_i$, 100 μg/mL SMP preincubated with MalNEt, and 12 mM ascorbate plus 3 mM TMPD as the oxidizable substrate. At zero MalNEt concentration, the oxidative phosphorylation activity of SMP oxidizing ascorbate + TMPD was 300 nmol min^{-1} mg^{-1}. At the highest concentrations of PAO and MalNEt shown, the succinate oxidase activity of the inhibitor-treated particles was at most inhibited by about 30%, while the ascorbate–TMPD oxidation rate was essentially unaffected by 8.5 mM MalNEt.

Results

Energy-Potentiated Uncoupling by Mono- and Dithiol Reagents. Figure 1 shows the results of three sets of experiments on the effects of PAO and MalNEt on the inhibition of oxidative phosphorylation and ATP–P_i exchange activities of SMP. The results with PAO are shown with triangles and solid lines. SMP at 0.9 mg/mL was incubated aerobically with the indicated concentrations of PAO for 30 min at 30 °C in the absence (△) and presence (▲, ▼) of succinate without (▲) and with (▼) 2,4-dinitrophenol. It was then transferred to an ice bath and assayed for ATP–P_i exchange activity. It is seen that in the absence of succinate, PAO had a small inhibitory effect on the ATP–P_i exchange activity of SMP, which was increased considerably when succinate was added to the incubation mixture. For example, at 0.44 mM PAO, the degrees of inhibition in the absence and presence of succinate were, respectively, 9% and 43%. Similar results were obtained when

[1] Abbreviations: AcPyADP, 3-acetylpyridine adenine dinucleotide phosphate; DCCD, dicyclohexylcarbodiimide; diamide, diazenedicarboxylic acid bis(dimethylamide); F_1 and CF_1, soluble ATPase from mitochondria and chloroplasts, respectively; F_0, membrane sector of the ATP synthase complex; OPDM, *o*-phenylenedimaleimide; OSCP, oligomycin sensitivity conferring protein; PAO, phenylarsine oxide; PCMPS, *p*-(chloromercuri)benzenesulfonate; MalNEt, *N*-ethylmaleimide; I_{50}, concentration of a reagent causing 50% inhibition or uncoupling; NBF-Cl, 4-chloro-7-nitrobenzofurazan; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; SMP, sonicated submitochondrial particles; AE-SMP, SMP extracted with ammonia–EDTA; EDTA, ethylenediaminetetraacetic acid; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

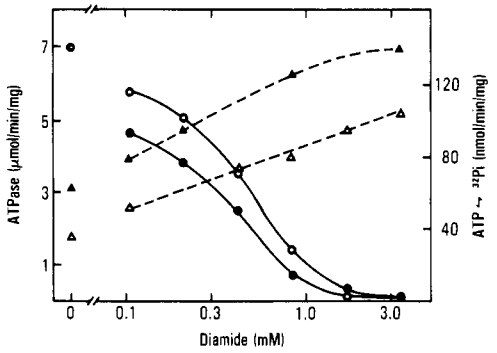

FIGURE 2: Effect of preincubation of SMP with diamide on ATP–$^{32}P_i$ exchange (circles) and ATPase (triangles) activities. SMP at 0.8 mg/mL was preincubated for 30 min at 30 °C with the indicated amounts of diamide in the presence (●, ▲) and absence (○, △) of 18 mM succinate under the conditions described in Figure 1. The ATP–$^{32}P_i$ exchange assay was also the same as in Figure 1, except that ATP concentration was 2 mM and SMP concentration was 80 μg/mL. ATPase activity was assayed at 30 °C in the presence of 8 μg of SMP/mL.

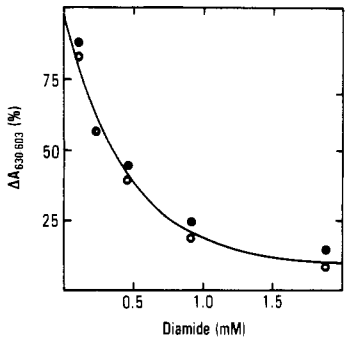

FIGURE 3: Effect of preincubation of SMP with diamide on respiration-induced membrane potential in the absence (○) and presence (●) of 50 μM DCCD. SMP at 1.0 mg/mL was incubated in the absence of added succinate with the indicated concentrations of diamide as described in Figure 2. Membrane potential was monitored by the absorbance change of oxonol VI at 630 minus 603 nm in a mixture containing 0.25 M sucrose, 50 mM Tris–acetate (pH 7.5), 4.6 mM $MgCl_2$, 1.78 μM oxonol VI, and 90 μg of SMP/mL. Membrane potential was induced by the combined addition of 18.2 mM ascorbate and 2.9 mM TMPD. In (●), the preincubated SMP was further treated with 50 μM DCCD. Identical results were obtained when instead of DCCD the particles were treated with 25 μg of oligomycin/mg. It has been shown elsewhere (Yagi et al., 1984) that as a monitor of membrane potential the decrease in the absorbance change of oxonol VI parallels the loss of oxidative phosphorylation activity of SMP when the system is challenged with incremental additions of an uncoupler.

the particles were incubated for 10 min at 30 °C (circles and dashed lines) with the indicated amounts of MalNEt and assayed for oxidative phosphorylation. Replacement in the incubation mixture of succinate with fumarate or malonate did not enhance the inhibitory effect of MalNEt or PAO, and addition of 2,4-dinitrophenol together with succinate (▼) abolished the succinate effect, thus indicating that sensitization of the oxidative phosphorylation activity of SMP toward inhibition by PAO and MalNEt was related to membrane energization as a consequence of succinate oxidation. The reason for the use of 2,4-dinitrophenol as uncoupler was because of the high I_{50} of this compound ($I_{50} \simeq 15$ μM) as compared to those of other uncouplers (Hatefi, 1975). In the incubation mixture, the concentration of 2,4-dinitrophenol was 540 μM, but in the oxidative phosphorylation assay, it was diluted to 5.4 μM, which caused less than 25% uncoupling when added to a control ATP–P_i exchange assay medium. The dotted lines in Figure 1 show the results of incubation of SMP with the indicated amounts of MalNEt for 30 min at 30 °C in the absence (□) and presence (■) of succinate. It is seen that the inhibition of oxidative phosphorylation (similar results were obtained for ATP–P_i exchange, not shown) was much greater when the particles were incubated with MalNEt for 30 min instead of 10 min and that at higher MalNEt concentrations the effect of succinate was considerably diminished. These results suggest that the effect of membrane energization as a result of succinate oxidation is not one of unmasking the targets for PAO and MalNEt but may be a consequence of making the targets more accessible to these reagents. Essentially similar results were obtained with $CdSO_4$, diamide, OPDM, and monobromobimane as inhibitors.

In addition to inhibiting ATP synthesis and ATP–P_i exchange, the above reagents also stimulated the oligomycin-sensitive ATPase activity of SMP (Figure 2) and decreased the ability of the particles to build up and maintain a membrane potential (Figure 3). Thus, the basic and common effect of the above reagents appears to be uncoupling [see also Siliprandi et al. (1974)]. In chloroplasts, internal cross-linking in the γ subunit caused by OPDM also resulted in uncoupling. In this case, addition of DCCD to OPDM-treated thylakoids restored their ability for proton uptake, which agreed with the finding that the site of OPDM modification was beyond the proton channel in the γ subunit of CF_1. However, in the case of SMP, addition of DCCD or oligomycin did not restore the ability of diamide-treated particles to maintain a membrane potential (Figure 3). Thus, although mitochondria and chloroplasts share the common feature of possessing thiol groups whose modification by appropriate reagents is energy potentiated and results in uncoupling, the location of these thiols may be different in the two systems. The dual facts (a) that modification of SMP by the above thiol reagents resulted in stimulation of oligomycin-sensitive ATPase activity and (b) that in some cases (e.g., modification by MalNEt) a transient membrane potential could be formed as a result of ATP hydrolysis indicate that the thiol modifications do not interfere with ATP hydrolysis and ATP-induced proton translocation through the ATP synthase complex. However, this does not mean that the lesion is in a part of the membrane other than the ATP synthase complex. Indeed, the same uncoupling phenomena described above with respect to SMP (i.e., inhibition of ATP–P_i exchange and loss of ability to maintain a membrane potential) were observed with preparations of the ATP synthase complex (complex V) when the latter were treated with any one of the above thiol reagents (data not shown).

The uncoupling by phenylarsine oxide, OPDM, $CdSO_4$, and diamide suggests the involvement of a dithiol, which the first three compounds would modify and diamide could oxidize to a disulfide. Thus, it might be expected that thiol compounds might reverse the uncoupling by diamide. It was found that dithiothreitol and dithioerythritol were only partially effective, while β-mercaptoethanol and dihydrolipoamide reversed the uncoupling by diamide. The results with dihydrolipoamide are shown in Figure 4. It is seen that diamide treatment of SMP resulted in 90% inhibition of ATP–P_i exchange and complete absence of a detectable membrane potential as measured by the oxonol VI absorbance change and that further treatment of the particles with about 1 mM dihydrolipoamide resulted in about 80% recovery of both the membrane potential and the ATP–P_i exchange activity. Mercaptoethanol was less potent than dihydrolipoamide; a comparable degree of recovery of ATP–P_i exchange activity required the addition of 100–150 mM mercaptoethanol. Diamide treatment of SMP also stimulated the ATPase activity from 2.2 to 5.3 μmol min^{-1}

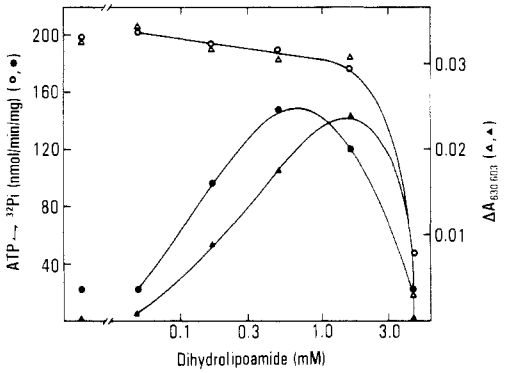

FIGURE 4: Reversal by dihydrolipoamide of the uncoupling effect of diamide as monitored by the ATP–$^{32}P_i$ exchange activity and the respiration-induced membrane potential of SMP. The particles were preincubated in the absence (○, △) and presence (●, ▲) of 1.8 mM diamide, as described in Figure 2, and then further treated for 30 min at 30 °C with the indicated amounts of dihydrolipoamide. The latter was generated from oxidized lipoamide by titration with dithiothreitol. The assays for ATP–$^{32}P_i$ exchange and the oxonol VI absorbance change at 630 minus 603 nm were the same as those in Figures 1 and 3, respectively, except that membrane potential was generated by addition of 1 mM ATP in the presence of 5 mM $MgCl_2$.

Table I: Reconstitution of ATP-Driven NAD Reduction by Succinate, Using AE-SMP, F_1, OSCP, Factor B, and Oligomycin[a]

expt	additions to AE-SMP	sp act.
1	none	10
	oligomycin	176
	factor B	194
2	none	17
	F_1	84
	F_1 + OSCP	97
	factor B	124
	F_1 + OSCP + factor B	143

[a] The assay media contained in 1.0 mL of 0.25 M sucrose, 50 mM Tris-sulfate (pH 7.5), 6 mM $MgCl_2$, 0.1% bovine serum albumin, 5 mM KCN, 10 mM succinate, 800 μM NAD, 10 mM phosphoenolpyruvate, 50 units of pyruvate kinase, 4 mM ATP, 126 μg of AE-SMP, and, where indicated, 19 ng of oligomycin, 9.25 μg of factor B, 40 μg of F_1, and 1.43 μg of OSCP. Specific activity is expressed as nanomoles of NAD reduced per minute per milligram of AE-SMP protein at 30 °C.

mg^{-1}. The stimulated ATPase activity was oligomycin sensitive, and subsequent treatment of SMP with 1.0 mM dihydrolipoamide reversed it to 3.4 μmol min^{-1} mg^{-1} (data not shown). When the particles were completely uncoupled with PCMPS or $CdSO_4$, addition of dihydrolipoamide up to 1.5 mM or more resulted in only 47% reversal of the PCMPS and 30% reversal of the $CdSO_4$ inhibition of ATP–P_i exchange activity. Reversal of $CdSO_4$ inhibition by 100–200 mM mercaptoethanol was also about 30%. It might be noted in Figure 4 that high concentrations (>2 mM) of dihydrolipoamide seemed to cause uncoupling. This effect, which is also brought about by treatment of SMP with 2,3-dimercaptopropanol [British anti-Lewisite (BAL)] and mercaptoethanol, is currently under study.

Is Factor B the Site of Uncoupling by Mono- and Dithiol Modifiers? It has been stated by Sanadi (1982) that factor B deficient particles as prepared by ammonia–EDTA extraction of SMP (AE-SMP) are not uncoupled. Our results indicate that they are uncoupled, but only partially and in a manner that can be repaired by addition of oligomycin. Preparations of SMP were depleted from factor B by multiple ammonia–EDTA extraction until their residual ATP-driven electron-transfer activity from succinate to NAD was ≤10% of the unextracted particles (this treatment also results in

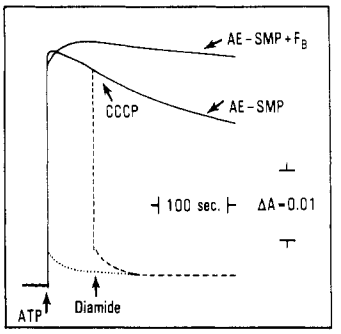

FIGURE 5: Effects of factor B or preincubation with diamide on ATP-induced static head membrane potential in AE-SMP. Where indicated, 2 μg of factor B or 1 μM CCCP was added to the assay mixture, or AE-SMP was preincubated as described in Figure 2 with 4.5 mM diamide. The assay conditions were the same as those in Figure 3, except that the reaction mixture contained 4.9 mM $MgCl_2$, 1.95 μM oxonol VI, and 123 μg of AE-SMP or diamide-treated AE-SMP. Membrane potential was generated by addition of 1.95 mM ATP.

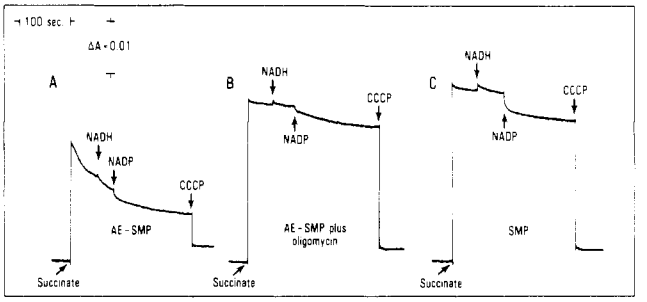

FIGURE 6: Membrane potential of AE-SMP under steady-state conditions. Membrane potential was monitored by the oxonol VI absorbance change as described in Figure 5 in the presence of 126 μg of either AE-SMP (trace A), AE-SMP plus 0.25 μg of oligomycin (trace B), or intact SMP (trace C) and 10 μg of rotenone/mL. Membrane potential was generated by addition of 10 mM succinate. Where indicated, 1.0 mM NADH, 1.0 mM NADP, and 10 μM CCCP were added.

partial removal of F_1). As before (Stiggall et al., 1979a), addition of factor B or low levels of oligomycin to the deficient particles caused near-complete restoration of the above activity (Table I, experiment 1). In the absence of added factor B or oligomycin, the deficient particles did form a respiration-induced membrane potential. However, the static head membrane potential was not stable and decayed slowly to a lower level (Figure 5). Addition of factor B, low levels of oligomycin, or F_1 ± OSCP resulted in considerable stabilization of the static head membrane potential (Figure 5) and restoration of ATP-driven electron transfer from succinate to NAD (Table I). When examined under steady-state conditions by coupling respiration-induced membrane energization to the transhydrogenase reaction, then the partially uncoupled state of the extracted particles was much more evident. These results are shown in Figure 6. Traces A and C show the effects of successive additions of succinate, NADH, NADP (at this point steady-state conditions are established), and CCCP, respectively, to factor B deficient and intact SMP. Even though energy utilization by the transhydrogenase reaction is very low [in NADH → NADP transhydrogenation, $[H^+]/[H^-] \simeq 1$, whereas in succinate oxidation, $[H^+]/(2e^-) = 6–8$], it is seen that as compared to SMP the extracted particles could only maintain a low level of steady-state membrane potential. However, in agreement with the data of Table I, it is seen in trace B of Figure 6 that addition of oligomycin to the extracted particles repaired their defect and resulted in the maintenance of a high level of steady-state

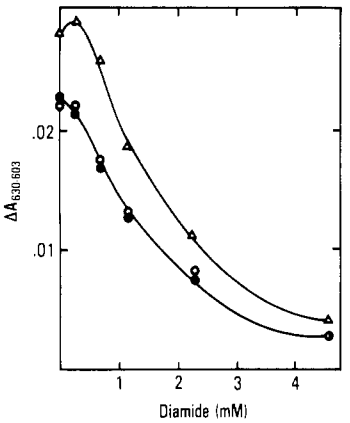

FIGURE 7: Uncoupling by diamide of AE-SMP particles (○) supplemented with F_1 + OSCP (●) or treated with low levels of oligomycin (△). AE-SMP at 25.5 mg/mL was preincubated with the indicated amounts of diamide as described in Figure 2. It was then diluted 10-fold with 0.25 M sucrose containing 10 mM Tris–acetate (pH 7.5) and kept on ice for assay. The assay mixture contained 0.25 M sucrose, 50 mM Tris–acetate (pH 7.5), 4.7 mM $MgCl_2$, 1.95 μM oxonol VI, and 117 μg of diamide-treated AE-SMP, and, where indicated, 23 μg of F_1 plus 2.5 μg of OSCP (●) per mL or 0.2 μg of oligomycin (△) per mg of AE-SMP. Membrane potential was generated by addition of 1.87 mM ATP.

membrane potential. These results indicate that the proton leakiness of factor B deficient particles is somewhere between F_1 and the site of oligomycin inhibition. Another indication of the partially uncoupled state of the deficient particles is that treatment of these particles with factor B can diminish their ATPase activity (Sanadi, 1982).

The above results suggest that multiple ammonia–EDTA extraction of SMP, even to the extent that reduces the rate of ATP-driven reverse electron transfer to <10% of the control, results in only partial removal of factor B, in addition to causing partial uncoupling. This is suggested by the fact that considerable activity can be restored to these particles by addition of low levels of oligomycin or F_1 + OSCP, which prevent the decay of the membrane potential. Thus, if we assume that factor B is definitely required for membrane energization by ATP hydrolysis, these results would indicate that the extracted particles still contain sufficient factor B to allow activity restoration once the membrane leakiness is repaired by addition of F_1 + OSCP or low levels of oligomycin. If this reasoning is correct, then the target for uncoupling by the thiol modifiers discussed above could be factor B. However, the existence of a separate dithiol target is supported by the fact that, unlike the partial uncoupling of factor B deficient particles (Figure 6), the uncoupling by thiol modifiers cannot be reversed by addition of oligomycin. In this regard, the following observations may be added. Factor B deficient particles could be further uncoupled when treated with diamide (Figures 5 and 7), $CdSO_4$, or PCMPS (results not shown), and addition to the deficient particles of F_1 ± OSCP, oligomycin, or factor B did not prevent the uncoupling by the above reagents. Also, addition to the deficient particles of factor B treated with diamide, MalNEt, or $CdSO_4$ did not cause further uncoupling, indicating that modified factor B does not behave as an uncoupler.

Inhibition of ATP Hydrolysis by Trialkyltin (or Aryltin) Compounds. Substituted tin compounds have been shown to inhibit membrane-bound ATPase activity in SMP and ATP synthase preparations. This inhibition is reversed by dithiols (Cain et al., 1977). Thus, here is yet another modifier of the ATP synthase complex whose inhibitory effect is specifically reversed by thiols, and the question is whether the target for

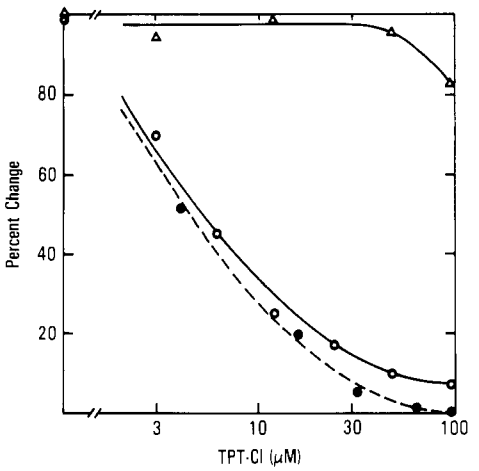

FIGURE 8: Effect of TPT-Cl concentration on the ATP–$^{32}P_i$ exchange (●) and ATPase (○) activities and the respiration-induced membrane potential (△) of SMP. The particles at 11 mg/mL in 0.25 M sucrose and 50 mM Tris–acetate (pH 7.5) were incubated with the indicated amounts of TPT-Cl for 30 min at 30 °C. They were then diluted 10-fold with sucrose–Tris buffer and kept on ice for assay. The assay conditions for ATP–$^{32}P_i$ exchange and ATPase activities were the same as those in Figures 1 and 2, respectively. Membrane potential was generated at 30 °C by the combined addition of 10 mM ascorbate plus 0.8 mM TMPD to a reaction mixture containing 0.25 M sucrose, 50 mM Tris–acetate (pH 7.5), 5 mM $MgCl_2$, 92 μg of TPT-Cl-treated SMP, and 1.95 μM oxonol VI and was monitored at 630 minus 603 nm.

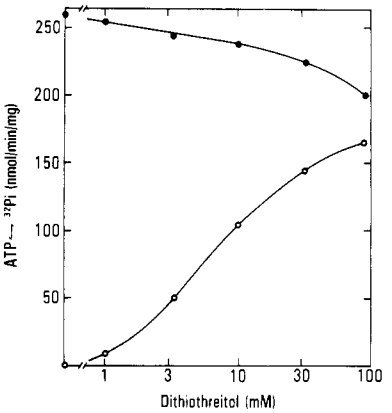

FIGURE 9: Reversal by dithiothreitol of the ATP–$^{32}P_i$ exchange activity of SMP inhibited by TPT-Cl. SMP at 8.9 mg/mL was incubated as described in Figure 8 in the absence (●) and presence (○) of 77 μM TPT-Cl. It was then diluted 10-fold with buffer, treated for 30 min at 30 °C with the indicated amounts of dithiothreitol, and assayed for ATP–$^{32}P_i$ exchange activity as described in Figure 1, except that the ATP concentration was 2 mM and the SMP concentration was 113 μg/mL.

the substituted tin compounds is related to factor B and the target for the thiol reagents discussed earlier. The answer to this question appears to be negative. Factor B extraction, or addition to deficient particles of factor B modified by thiol reagents, does not inhibit ATPase activity, and the thiol reagents discussed above stimulate the ATPase activity of SMP 3–4-fold. By contrast, TPT-Cl at <10 μM causes 50% inhibition of the ATPase activity of SMP or factor B deficient particles. At concentrations which completely inhibit ATPase and ATP–P_i exchange activities, TPT-Cl does not cause uncoupling (Figure 8), and TPT-inhibited particles can still be uncoupled by addition of thiol and dithiol modifiers (results not shown). Moreover, the inhibition of ATPase activity by TPT-Cl is effectively reversed by dithiothreitol (Figure 9) (also by dihydrolipoamide or mercaptoethanol; data not shown), whereas the uncoupling by diamide is not reversed more than

25–40% by high concentrations of dithiothreitol or dithioerythritol, even after prolonged incubation.

Discussion

Three apparently different types of modification of the mitochondrial ATP synthase result in inhibition of oxidative phosphorylation, all of which can be reversed by treatment with thiol compounds. These modifications are as follows:

(1) SMP and ATP synthase preparations can be rendered deficient with respect to factor B, which is a water-soluble, low molecular weight protein containing an essential vicinal dithiol. Removal (apparently partial) of factor B from SMP by multiple extraction of the particles with ammonia–EDTA results in the loss of energy-transfer capability to and from F_1-ATPase. Thus, oxidative phosphorylation, ATP–P_i exchange, ATP-driven transhydrogenation, and ATP-driven uphill electron transfer from succinate to NAD are all impaired by this treatment. Addition of factor B restores these functions (Stiggall et al., 1979a). Treatment of factor B with Cd^{2+}, phenylarsine oxide, or diamide renders factor B ineffective in restoring the above energy-transfer functions of factor B deficient particles. This modification of factor B activity can be reversed by addition of thiol compounds, such as dithiothreitol or BAL (Sanadi, 1982). The respiration-induced membrane potential of factor B deficient SMP slowly decays to a lower static head level, and the steady-state level of membrane potential maintained by these particles is very low when respiration is coupled to the transhydrogenase reaction. These defects are repaired by addition of factor B or oligomycin (also partially by addition of F_1). Addition of factor B also lowers the ATPase activity of the deficient particles. The role of factor B in energy transfer to and from F_1-ATPase is not known, but the above results suggest that its partial removal by ammonia–EDTA is accompanied by partial uncoupling in a manner that is repairable by addition of oligomycin.

(2) Trialkyltin (or aryltin) compounds inhibit ATP synthesis and hydrolysis in SMP and ATP–P_i exchange and ATP hydrolysis in ATP synthase preparations, but do not inhibit the hydrolytic activity of isolated F_1-ATPase. TPT-Cl does not uncouple SMP at concentrations which completely inhibit ATP hydrolysis, but TPT-inhibited particles can be uncoupled by treatment with thiol reagents. The inhibitory effect of TPT-Cl is reversed by mono- and dithiols, such as mercaptoethanol, dithiothreitol, or dihydrolipoamide. Charged monothiols such as reduced glutathione and cysteine are poorly effective. The locus in F_0 and the nature of the group modified by the substituted tin compounds are not known. The reversibility of the inhibition by dithiols has suggested that the group modified by these compounds might be a thiol. However, this conclusion may not be correct. NBF-Cl is another reagent which inhibits ATPase activity, and this inhibition is readily reversed by dithiothreitol. In the case of NBF-Cl, it is known that this compound reacts with a tyrosyl residue in the β subunit of F_1-ATPase. Indeed, the possibility that in rat hemoglobin triethyltin might react with histidyl residues has been advanced (Rose, 1969), whereas in the case of yeast hexokinase, which is inhibited by triethyltin bromide, it has been concluded that neither cysteine nor histidine is liganded to the inhibitor (Siebenlist & Taketa, 1983).

(3) A number of mono- and dithiol modifiers, including MalNEt, PCMPS, PAO, Cd^{2+}, diamide, OPDM, and monobromobimane, uncouple SMP and complex V and stimulate their ATPase activities, particularly that of SMP. They also uncouple SMP deficient in factor B (AE-SMP), or F_1-depleted ASU particles ± F_1 and OSCP. These results indicate that the site of uncoupling is in F_0 and not in F_1. In contrast to AE-SMP, addition of oligomycin or DCCD to these uncoupled particles does not restore coupling, which further suggests that the lesion is not on the F_1 (matrix) side of the block points of oligomycin and DCCD in the proton channel. Furthermore, the uncoupling is energy potentiated, suggesting that access to the site of uncoupling and/or the reactivity of the groups modified are altered by energy-induced conformational changes of F_0. A similar energy-potentiated uncoupling occurs when chloroplast thylakoids are treated with OPDM (Weiss & McCarty, 1977). However, in this case, the modification effected by OPDM was shown to be a cross-linking within the γ subunit of CF_1. The uncoupling of SMP by the appropriate thiol reagents is reversible. The uncoupling by diamide was reversed 80–90% by dihydrolipoamide or mercaptoethanol and only partially by dithiothreitol and dithioerythritol. The uncoupling by PCMPS and Cd^{2+} was also reversed by dihydrolipoamide up to 50% and 30%, respectively. The reversal of the uncoupling effect of diamide was shown to be accompanied by lowering of the diamide-stimulated ATPase activity, which is consistent with the controlled rate of ATP hydrolysis in coupled membranes.

It is clear from the above discussion that the effect of TPT-Cl is distinctly different from the other two, and whether the target for TPT-Cl is a thiol or some other protein residue is not known. The phenomena discussed in (1) and (3) may or may not be related, however. Our experiments suggest that ammonia–EDTA extraction of SMP results in only partial extraction of factor B and that the factor B deficient particles are partially uncoupled. The fact that such partial uncoupling, as judged from the unstable static head and the low steady-state level of the membrane potential, can be associated with >90% inhibition of ATP-driven reverse electron transfer from succinate to NAD has been discussed elsewhere (Yagi et al., 1984). Thus, it is possible that both the removal of factor B and the in situ modification of factor B by mono- and dithiol modifiers result in uncoupling and that the access of modifiers to this site is facilitated by energy-induced conformational changes of the membrane. On the other hand, it is also possible that the target for uncoupling by thiol and dithiol modifiers is different from factor B. In that case, energy transfer in the ATP synthase complex would involve, among other things, two different essential dithiols. The latter possibility is supported by the fact that partial uncoupling of factor B deficient particles is repairable by oligomycin, whereas uncoupling by mono- and dithiol modifiers is not.

What is rather intriguing is that the above essential dithiols may be unique to the mitochondrial ATP synthase. The membrane sectors (F_0) of the ATP synthases of *Escherichia coli* and thermophilic bacterium PS3 are each composed of three polypeptides. The F_0 polypeptides of *E. coli* contain only a single cysteine residue in subunit b (Nielsen et al., 1981), and in the thermophilic bacterium F_0, the cysteine content is only about 0.48 mol % (Kagawa et al., 1976). Neither ATP synthase appears to have a polypeptide analogous to factor B, and whether they are uncoupled by mono- and dithiol reagents is not known. Even in the case of chloroplasts, the energy-potentiated modification of the γ subunit and the consequent uncoupling are not entirely analogous to the mitochondrial uncoupling. In chloroplasts, only the bifunctional maleimides, such as OPDM, cause uncoupling, apparently by internal cross-linking and structural modification of the γ subunit, while the monofunctional MalNEt inhibits photophosphorylation but does not uncouple. By contrast, in mitochondria, both mono- and bifunctional maleimides, as well as PCMPS and several

dithiol modifiers, cause uncoupling. Thus, it is possible that the mechanism of energy transfer in the mitochondrial ATP synthase may be different from those in the bacterial and chloroplast systems, and this may be reflected in the fact that the mitochondrial ATP synthase appears to contain three to five more polypeptides than its bacterial counterpart.

Another intriguing observation, briefly reported in this paper, is that dihydrolipoamide at concentrations up to 1.2 mM reversed the uncoupling caused by treatment of SMP with diamide, but at higher concentration itself appeared to uncouple. BAL and mercaptoethanol also uncouple at high concentrations. These results seem to suggest the involvement of two or more sets of dithiol–disulfides in the mitochondrial ATP synthase, whose oxidation–reduction states are essential to energy transfer.

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Registry No. PAO, 637-03-6; NBF-Cl, 10199-89-0; TPT-Cl, 639-58-7; MalNEt, 128-53-0; PCMPS, 554-77-8; OPDM, 13118-04-2; Cd, 7440-43-9; ATPase, 9000-83-3; ATP synthase, 37205-63-3; diamide, 10465-78-8; monobromobimane, 71418-44-5; succinic acid, 110-15-6; dithiothreitol, 3483-12-3; dithioerythritol, 6892-68-8; β-mercaptoethanol, 60-24-2; dihydrolipoamide, 3884-47-7; oligomycin, 1404-19-9.

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