Characterization of phosphate efflux pathways in rat liver mitochondria

Ronald S. KAPLAN and Peter L. PEDERSEN*

Laboratory for Molecular and Cellular Bioenergetics, Department of Physiological Chemistry, The Johns Hopkins University School of Medicine, Baltimore, MD 21205, U.S.A.

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ATP hydrolysis catalysed by the H^+ -ATPase of intact mitochondria can be induced by addition of ATP in the presence of valinomycin and KCl. This leads to an increase in intramitochondrial P_i and therefore allows investigation of potential P_i efflux pathways in intact mitochondria. Combining this approach with the direct measurement of both internal and external P_i , we have attempted to determine whether P_i efflux occurs via an atractyloside-sensitive transporter, by the classical operation of the P_i/H^+ and P_i /dicarboxylate carriers, and/or by other mechanisms. Initial experiments re-examined the evidence that led to the current view that one efflux pathway for P_i is an atractyloside-sensitive ATP/ADP,0.5 P_i transporter. No evidence was found in support of this efflux pathway. Rather, atractyloside-sensitivity of the low rate of P_i efflux observed in previous studies (oligomycin present) was accounted for by ATP entry on the well known ATP/ADP transport system followed by hydrolysis of ATP and subsequent P_i efflux. Thus, under these conditions, where ATP hydrolysis is not completely inhibited, P_i efflux becomes atractyloside sensitive most likely because this inhibitor blocks ATP entry, not because it directly inhibits P_i efflux. Substantial efflux of P_i from rat liver mitochondria is observed on generation of high levels of matrix P_i by ATP hydrolysis induced by valinomycin and K^+ (oligomycin absent). A portion of this efflux can be inhibited by thiol-specific reagents at concentrations that normally inhibit the P_i/H^+ and P_i /dicarboxylate carriers. However, a significant fraction of efflux continues even in the presence of *p*-chloromercuribenzoate, *N*-ethylmaleimide plus *n*-butylmalonate or mersalyl. The mersalyl-insensitive P_i efflux, which is also insensitive to carboxyatractyloside, is a saturable process, thus suggesting carrier mediation. During this efflux the mitochondrial inner membrane retains considerable impermeability to other low-molecular-weight anions (i.e., malate, 2-oxoglutarate). In conclusion, results presented here rule out an atractyloside-sensitive ATP/ADP,0.5 P_i transport system as a mechanism for P_i efflux in rat liver mitochondria. Rather P_i efflux appears to occur on the classical P_i/H^+ transport system as well as via a mersalyl-insensitive saturable process. The inhibitor-insensitive P_i efflux may occur on a portion of the P_i/H^+ carrier molecules that exist in a state different from that normally catalysing P_i influx. Alternatively, a separate P_i efflux carrier may exist.

P_i moves across the mitochondrial inner membrane via at least two distinct transport systems. One system (the P_i carrier) catalyses a P_i/H^+ symport process, is dependent on a transmembrane

pH gradient and can be inhibited by NEM, mersalyl and *p*-CMB (Fonyó & Bessman, 1968; Tyler, 1969; Coty & Pedersen, 1974, 1975; for review, see Fonyó, 1978, 1979). The other system, the P_i /dicarboxylate carrier, catalyses a P_i /dicarboxylate antiport, is insensitive to NEM and can be inhibited by mersalyl, *p*-CMB or the substrate analogue *n*-butylmalonate (Meyer & Tager, 1969; Meijer *et al.*, 1970; Quagliariello & Palmieri, 1972; Coty & Pedersen, 1974). A current point of controversy focuses on whether the P_i carrier catalyses both phosphate influx and efflux or whether an additional

Abbreviations used: NEM, *N*-ethylmaleimide; *p*-CMB, *p*-chloromercuribenzoic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; DAPP, adenosine(5')pentaphospho(5')adenosine [P^1P^5 -di-(adenosine-5')pentaphosphate]; CAT, carboxyatractyloside; BSA, bovine serum albumin.

* To whom all correspondence and reprint requests should be sent.

carrier is required for the efflux pathway (Azzone *et al.*, 1976; Wehrle *et al.*, 1978; Wehrle & Pedersen, 1981). This uncertainty has arisen from two types of evidence. First, inhibitor studies indicate that although NEM and certain organic mercurials are efficient inhibitors of P_i influx (Fonyó & Bessman, 1968; Tyler, 1969; Meijer *et al.*, 1970; Coty & Pedersen, 1974, 1975; Fonyó & Vignais, 1980), their ability to inhibit efflux is more limited and varies considerably depending on the incubation conditions (i.e. electron transport inhibitors, uncouplers, valinomycin, Mg^{2+} etc.) (Siliprandi *et al.*, 1975; Azzone *et al.*, 1976; Reynafarje & Lehninger, 1978; Fonyó & Vignais, 1979; Tyler, 1980). Secondly, studies with intact mitochondria and purified inverted inner-membrane vesicles indicate that the energy-coupling mechanisms involved may differ depending on the direction of phosphate transport (Coty & Pedersen, 1974; Wehrle *et al.*, 1978; Wehrle & Pedersen, 1981).

Recently, yet another carrier has been proposed for P_i efflux (Reynafarje & Lehninger, 1978). This carrier is purported to be an ATP/ADP, 0.5 P_i transport system, perhaps sharing a common peptide with the classical ATP/ADP carrier. Evidence for this transport system derives mainly from the finding that atractyloside inhibits ATP-generated P_i efflux from mitochondria under conditions where ATP hydrolysis is thought to be completely inhibited by oligomycin. Although two groups (Fonyó & Vignais, 1979; Tyler, 1980) have argued against the validity of this hypothesis, the original experimental observations have not been adequately explained.

The objectives of the present study were 2-fold. First, by direct measurement of both internal and external P_i , it was essential to carefully re-examine the conditions that led to the proposal for an atractyloside-sensitive ATP/ADP, 0.5 P_i transporter (Reynafarje & Lehninger, 1978). Under these conditions low amounts of matrix P_i are generated. Secondly, it seemed equally important to examine P_i efflux under conditions where high levels of matrix P_i are generated in order to obtain a more complete picture of the P_i efflux process. Specifically, we wanted to examine the effect of a variety of transport inhibitors on the P_i efflux process to establish whether efflux occurred on the well characterized P_i/H^+ and P_i /dicarboxylate transport systems and/or by additional mechanisms. Results of experiments designed to address these objectives are described in detail below.

Experimental procedures

Materials

Mersalyl acid, rotenone, valinomycin, atractyloside (K^+ salt), carboxyatractyloside (dipotassium

salt), oligomycin, antimycin A, *p*-CMB, NEM, DAPP (Li^+ salt), BSA (essentially fatty acid-free) were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. ADP (monopotassium salt) and lactate dehydrogenase (rabbit muscle) were supplied by Boehringer Mannheim Biochemicals, Indianapolis, IN, U.S.A. Finally, *n*-butylmalonic acid was obtained from Aldrich Chemical Co., Milwaukee, WI, U.S.A., ATP (disodium salt) from P.L. Biochemicals, Milwaukee, WI, U.S.A., and sodium arsenite from Fisher Scientific Co., Silver Spring, MD, U.S.A.

Isolation of mitochondria

Rat liver mitochondria were isolated from male CD rats (retired breeders from Charles River Breeding Laboratories, Wilmington, MA, U.S.A.) employing a procedure similar to that previously described (Pedersen *et al.*, 1978). A rat that had been starved overnight was killed by decapitation, and bled under cold water. The liver was excised and placed into ice-cold 0.25 M-sucrose. All subsequent steps were carried out in 0.25 M-sucrose at 0–4°C. The excised liver was cut into small pieces, washed several times and homogenized (two strokes and then five strokes; Thomas Tissue Grinder, type C). The homogenate was then diluted to 100 ml and centrifuged at 750 *g* for 12 min (Sorvall, RC2-B centrifuge; SS-34 rotor). After centrifugation, the white layer at the top of the supernatant was discarded. The remaining supernatant was then centrifuged at 6800 *g* for 12 min. The resulting pellet was resuspended with a cold finger, diluted to 50 ml and centrifuged at 9800 *g* for 12 min. This pellet was then resuspended as before, diluted to 25 ml and centrifuged as above. The final pellet was resuspended by hand in 1–2 ml of 0.25 M-sucrose employing a Thomas Tissue Grinder (type A).

Mitochondrial protein was determined via a modified biuret procedure (Gornall *et al.*, 1949; Tyler, 1969). The final assay mixture contained 0.25–4 mg of mitochondrial protein, 1.7 mM-sucrose, 0.267% (w/v) sodium cholate, 6.7% (w/v) NaOH and 0.1% (w/v) $CuSO_4$. After the $CuSO_4$ addition, the colour development proceeded for 5–10 min, after which the absorbances were read at 540 nm. BSA was employed as the standard.

Determination of *P/O* and acceptor control ratios

P/O and acceptor control ratios were determined by a procedure similar to that of Schnaitman & Greenawalt (1968). The reaction mixture contained 212 mM-mannitol, 66 mM-sucrose, 2.4 mM- KH_2PO_4 , 1.9 mM-Hepes, 0.48 mM-EDTA, pH 7.4; the mixture also contained 1.7 mM- $MgCl_2$, 0.69 mg of BSA/ml, either 5 mM-succinate or 10.4 mM- β -hydroxybutyrate, and 2.93 mg of mitochondrial protein. Reactions were run at 25°C and triggered by the sequential

addition of mitochondria and 0.2–0.45 μ mol of ADP. The incubation volume at this point was 1.98 ml and was considered to be the final volume. Usually, several ADP additions were made per trace. O₂ consumption was measured by a YSI Clark oxygen electrode and P/O as well as acceptor control ratios were determined graphically (Estabrook, 1967).

Determination of P_i

P_i was measured at room temperature by a procedure modified from that of Baginski *et al.* (1974) as described below. A solution (0.5 ml) of 23.4% (w/v) trichloroacetic acid/4.68% (w/v) L-ascorbic acid was added to 0.9 ml of a sample that was dissolved in 0.89 M-HClO₄. Ammonium molybdate (0.7 ml; 1.0%, w/v) was then added, followed within 3 min by the addition of 1.4 ml of a 2% (w/v) sodium arsenite/2% (w/v) citric acid reagent (adjusted to pH 7.6 with NaOH). At least 15 min were allowed for colour development and absorbances were read at 840 nm on a Beckman DU spectrophotometer (Gilford Photometer; model 252). Employing this procedure, P_i levels of at least 4–200 nmol could be determined and the final absorbance values were stable for at least several hours. In all P_i transport experiments, internal controls were run in order to quantify the minimal effects of the transport incubation constituents on the P_i assay absorbance values.

Determination of P_i appearance in the intramitochondrial and extramitochondrial volumes

Two procedures were employed to measure the appearance of P_i in the intramitochondrial and extramitochondrial volumes. These procedures, which differed primarily in the chemical composition of the incubation mixtures and in the controls employed, are described below.

Procedure 1 was used to measure the atractyloside effect on P_i appearance during the oligomycin-insensitive ATP hydrolysis reaction (Fig. 1). The reaction mixture contained Buffer A (120 mM-LiCl/4.0 mM-KCl/3.0 mM-potassium HEPES/1.0 mM-EGTA, pH 7.0), 20 μ M-DAPP (an inhibitor of adenylate kinase; Lienhard & Secemski, 1973), 0.6 nmol of rotenone/mg of protein, 4 μ g of oligomycin/mg of protein, 40 nmol of NEM/mg of protein, 24.8 mg of mitochondrial protein (added later), 200 ng of valinomycin/mg of protein (added later) and 0.25 mM-ATP (added later). Where indicated (Fig. 1b), 0.25 mM-attractyloside was present. Incubations were performed at 25°C, and were triggered by the addition of mitochondria, followed 90 s later by valinomycin, at which point the final volume was 7.5 ml. Before the addition of 0.25 mM-ATP at 300 s, control portions were removed. Experimental portions were subsequently removed at various times after the addition of ATP. All

portions were placed into centrifuge tubes that already contained 4 μ g of oligomycin/mg of protein and 1.9 nmol of CAT/mg of protein in Buffer A (pH 7). Additionally, ATP was added to the control tubes so that the final ATP concentration (0.20 mM) was equivalent in all tubes. After a 2 min centrifugation (Eppendorf Centrifuge, model 5412) extramitochondrial and intramitochondrial P_i were determined from the resulting supernatants and pellets respectively, as follows. Each fraction was extracted with 1 M-HClO₄, recentrifuged for 2 min and the P_i in the resulting supernatant was determined. The appearance of P_i with time was calculated by subtracting the mean control value from each experimental value. This subtraction corrected for the P_i initially present in the incubation mixtures as well as for any P_i production and/or transport that may have occurred subsequent to the timed ATP hydrolysis reaction.

Procedure 2 was used to measure the effect of several inhibitors (Figs. 2–5) on P_i appearance during the high-magnitude ATP hydrolysis reaction. The reaction mixture consisted of Buffer B (0.12 M-LiCl/22 mM-KCl/3.0 mM-potassium HEPES/0.5 mM-EGTA; pH 7.0), 20 μ M-DAPP, 0.8 nmol of rotenone/mg of protein, 0.2 nmol of antimycin/mg of protein, the inhibitors indicated in the appropriate Figure legends, 20 mg of mitochondrial protein (added later), 200 ng of valinomycin/mg of protein (added later) and 1.0 mM-ATP (added later). Before use, NEM and *p*-CMB stock solutions were assayed spectrophotometrically assuming ϵ values of 0.62 mM⁻¹.cm⁻¹ and 16.9 mM⁻¹.cm⁻¹ at wavelengths of 305 nm and 232 nm respectively (Boyer, 1954; Riordan & Vallee, 1967). Incubations were performed at 25°C in a final volume of 7.9 ml and were triggered by the addition sequence described in Procedure 1. Also, the removal of control and experimental portions was performed as described above. All portions were placed in centrifuge tubes that already contained 4.0 μ g of oligomycin/mg of protein and 2.4 nmol of CAT/mg of protein in Buffer B (pH 7). Portions were then centrifuged and subsequent steps were as described in Procedure 1. An additional control was performed in which ATP was diluted with deionized water to the same final concentration that was present in the incubation mixture. Portions from this solution were then subjected to the same procedure as for the experimental portions. The appearance of P_i was calculated by subtracting the sum of the P_i present in the ATP control (mean) and the control portion (mean) from the experimental portion values.

Determination of P_i efflux

The incubation mixtures contained Buffer B, 20 μ M-DAPP, 0.8 nmol of rotenone/mg of protein, 0.2 nmol of antimycin/mg of protein, 80 nmol of

mersalyl/mg of protein, 10mg of mitochondrial protein (added later), 200ng of valinomycin/mg of protein (added later) and 1.2mM-ATP (added later). Incubations were performed at 25°C and were initiated by the addition of mitochondria. Valinomycin was added 90s later, at which point the final volume was 4.0ml. After the removal of a control portion at 140s, ATP hydrolysis was initiated at 180s by the addition of 1.2mM-ATP. A portion was removed at 300s and ATP hydrolysis was then inhibited at 350s by the addition of an inhibitor mix (4µg of oligomycin/mg of protein/100µM-DAPP/1.0mM-EDTA/Buffer B, and, where indicated, 3nmol of CAT/mg of protein). The efflux of internal P_i was then measured as follows. Experimental portions were removed from the reaction mixture at various times. All portions were placed in centrifuge tubes that contained 3nmol of CAT/mg of protein (final concentration), 4µg of oligomycin/mg of protein (excludes the oligomycin present in the reaction mixture) and Buffer B (pH 7). Additionally, sufficient ATP was added to the control centrifuge tube so that the final ATP concentration (0.62mM) was the same as in the experimental tubes. Once in the centrifuge tubes, the portions were immediately centrifuged for 1min. The resulting supernatants were discarded and the pellets were extracted and assayed for internal P_i as described in Procedure 1.

Glutamate dehydrogenase and fumarase assays

Glutamate dehydrogenase activity was assayed by monitoring the oxidation of NADH at 340nm (Brdiczka *et al.*, 1968; Schmidt, 1974). The reaction mixture contained 47mM-triethanolamine hydrochloride/2.4mM-EDTA, pH 8.0, 40mM-(NH₄)₂SO₄, 0.2mM-NADH, 0.98mM-ADP (pH 7), 200mM-sucrose and 2–3µg of mitochondrial protein. Endogenous pyruvate was determined via the addition of 1.38µkat of lactate dehydrogenase (rabbit muscle). The main reaction was then triggered by the addition of 7mM-2-oxoglutarate (pH 7). Incubations were performed at 25°C in a final volume of 1.5 ml.

Fumarase activity was measured spectrophotometrically (Hill & Bradshaw, 1969) by following the production of fumarate ($\epsilon = 1.45\text{mM}^{-1}\cdot\text{cm}^{-1}$) at 250nm (Bock & Alberty, 1953). The reaction

mixture consisted of 250mM-sucrose, 50mM-sodium phosphate buffer, pH 7.3, approx. 0.2–0.3mg of mitochondrial protein and 25mM-malate, pH 7.3. Reactions were performed at 25°C in a final volume of 3.0ml.

For both assays lubrol activation was accomplished by incubating mitochondria with 1mg of lubrol/mg of mitochondrial protein for at least 15 min at 0°C.

Results

Characteristics of mitochondria isolated in 0.25M-sucrose

Initial experiments focused on a re-examination of the evidence that led to the proposal (Reynafarje & Lehninger, 1978) that an atractyloside-sensitive ATP/ADP,0.5 P_i exchange occurs in rat liver mitochondria. Accordingly, the mitochondrial isolation and the P_i efflux studies were conducted employing conditions that were nearly identical with those used previously (Reynafarje & Lehninger, 1978). Thus mitochondria were isolated and suspended in a 0.25 M-sucrose medium that was not buffered or pH-adjusted. Table 1 depicts several properties of mitochondria isolated in this medium. High acceptor control ratios were obtained with either succinate or β -hydroxybutyrate as the respiratory substrate. Therefore, these mitochondria were considered to be functionally intact and relatively undamaged by the isolation procedure (Chappell & Hansford, 1972). Also, the observed P/O ratios approached the expected values of 2 with succinate and 3 with β -hydroxybutyrate, suggesting the normal operation of the three coupling sites that comprise oxidative phosphorylation (Lehninger, 1964). Finally, the endogenous phosphate levels were within the range of the values normally found with freshly isolated mitochondria (i.e. 10–30nmol of P_i /mg of protein) (Coty & Pedersen, 1974; Reynafarje & Lehninger, 1978). In summary, mitochondria isolated as described above appear to be in good physical condition and suitable for transport studies.

The effect of atractyloside on P_i distribution

A main piece of evidence leading to the proposal of an ATP/ADP, P_i transporter was the observation

Table 1. *P/O and acceptor control ratios and endogenous P_i values for mitochondria isolated in unbuffered 0.25M-sucrose*
Data were obtained as described in the Experimental procedures section and are means $\pm$ s.d. Numbers in parentheses represent the total number of repetitions of a given incubation. All results were obtained from at least three mitochondrial preparations. Abbreviation: ACR, acceptor control ratio.

Succinate		β -Hydroxybutyrate		Endogenous P_i (nmol/mg)
P/O	ACR	P/O	ACR	
1.7 $\pm$ 0.1 (12)	5.3 $\pm$ 0.6 (12)	2.7 $\pm$ 0.1 (9)	7.3 $\pm$ 2.9 (9)	14.2 $\pm$ 1.7 (12)

that in the presence of valinomycin, KCl, ATP, oligomycin and NEM a slow appearance of P_i occurred in the extramitochondrial volume, which could be substantially inhibited by atractyloside (Reynafarje & Lehninger, 1978). It was assumed that under these incubation conditions, ATP hydrolysis was totally abolished by oligomycin and therefore the appearance of external P_i was due solely to P_i transport. Accordingly, the atractyloside effect was taken to be an inhibition of a P_i efflux pathway. Since the P_i/H⁺ carrier was assumed to be blocked by NEM, a distinct atractyloside-sensitive P_i carrier was proposed. Also, since ATP disappearance paralleled the appearance of ADP and 0.5 P_i in the extramitochondrial volume it was thought that the newly proposed carrier catalysed an ATP/ADP,0.5P_i exchange.

To assess this proposal (Reynafarje & Lehninger, 1978) more closely, we have repeated the critical experiments (Fig. 1) but have employed a higher concentration of oligomycin (4 µg/mg of protein versus 1 µg/mg of protein). Also, in contrast with the studies described above, we have directly measured both intramitochondrial and extramitochondrial P_i. Fig. 1(a) clearly demonstrates that in the presence of a high concentration of oligomycin, some ATP hydrolysis continues. Approx. 58% of the additional P_i formed effluxes into the extramitochondrial volume via an unknown NEM-insensitive pathway. The remaining 42% is retained within the mitochondrial matrix. Thus, the P_i release observed in Fig. 1(a) and by other investigators (Reynafarje & Lehninger, 1978) appears to be due to both a hydrolysis reaction and a subsequent transport process. Fig. 1(b) shows that atractyloside causes a large drop in the rate of total P_i production (i.e. ATP hydrolysis). Under these conditions, only a slight

increase in external P_i occurs (56% less than that observed in Fig. 1a) and internal P_i levels actually decrease.

The most straightforward interpretation of these results would be to invoke the well-documented atractyloside inhibition of the classical ATP/ADP carrier (Pfaff *et al.*, 1965; Winkler *et al.*, 1968; Vignais *et al.*, 1973). Thus, atractyloside prevents ATP from entering the mitochondrial matrix and thereby causes a decrease in the observed oligomycin-insensitive ATP hydrolysis. Accordingly, internal P_i levels no longer rise and in fact decrease slightly. Also, since the level of transportable substrate (internal P_i) drops, the rate of appearance of external P_i (i.e. the rate of efflux) is markedly reduced. Similar findings were obtained when atractyloside was replaced by carboxyatractyloside (5 µM).

In summary, the data in Fig. 1 and previously published results (Reynafarje & Lehninger, 1978) are most simply explained via the inhibitory action of either atractyloside or carboxyatractyloside on the classical ATP/ADP carrier (Pfaff *et al.*, 1965; Winkler *et al.*, 1968; Vignais *et al.*, 1973). The data do not suggest the existence of an atractyloside (or CAT)-sensitive ATP/ADP,0.5P_i carrier. This conclusion is corroborated by additional experiments discussed below, which show that P_i efflux resulting from high matrix P_i levels is also insensitive to carboxyatractyloside.

The effect of various inhibitors on the efflux of intramitochondrial P_i generated via valinomycin/K⁺-stimulated ATP hydrolysis

Controversy exists within the literature concerning the ability of several inhibitors of the P_i influx pathway (e.g. NEM, mersalyl, p-CMB etc.) (Fonyó

Fig. 1. The effect of atractyloside on intramitochondrial and extramitochondrial P_i appearance during oligomycin-insensitive ATP hydrolysis

Reactions were performed as described in the Experimental procedures section. Results in (b) were from incubations that contained 0.25 mM-atractyloside before the addition of mitochondria. Results in (a) are for experiments in the absence of atractyloside. All data were obtained from four separate mitochondrial isolations. Total P_i was calculated as the sum of internal P_i and external P_i. □, Intramitochondrial P_i; △, extramitochondrial P_i; ○, calculated total P_i.

& Bessman, 1968; Tyler, 1969; Meijer *et al.*, 1970; Coty & Pedersen, 1974, 1975; Fonyó & Vignais, 1980) to inhibit P_i efflux (Siliprandi *et al.*, 1975; Azzone *et al.*, 1976; Reynafarje & Lehninger, 1978; Fonyó & Vignais, 1979; Tyler, 1980). We have measured the effect of these inhibitors on the efflux of the intramitochondrial P_i generated via the valinomycin/ K^+ -induced ATP hydrolysis. Fig. 2(a) shows that in the absence of transport inhibitors, a large valinomycin/ K^+ -induced ATP hydrolysis occurs. Nearly all the P_i formed effluxes into the extramitochondrial space. Fig. 2(b) demonstrates that a high concentration of oligomycin ($10\mu\text{g}/\text{mg}$ of protein) markedly reduces the level of ATP hydrolysis. It is therefore concluded that the mitochondrial inner membrane F_1 -ATPase is responsible for the high-magnitude P_i production depicted in Fig. 2(a). Fig. 3(a) shows that pre-incubation with NEM, an irreversible inhibitor of P_i influx on the P_i/H^+ carrier (Coty & Pedersen, 1975; Fonyó, 1978, 1979), results in the retention of 37% of the total P_i formed within the mitochondrial matrix. It should be noted that we employed a concentration of NEM ($40\text{nmol}/\text{mg}$ of protein) that has been shown in our earlier studies (Coty & Pedersen, 1975) to inhibit P_i influx nearly completely. Thus, even when the influx pathway is blocked, the majority of P_i efflux continues.

An attempt was made to completely inhibit P_i efflux via the addition of a high concentration ($80\text{nmol}/\text{mg}$ of protein) of mersalyl, a reversible inhibitor of influx on both the P_i/H^+ and $P_i/\text{dicarboxylate}$ carriers (Meijer *et al.*, 1970; Quagliariello & Palmieri, 1972; Siliprandi *et al.*, 1975; Fonyó & Vignais, 1980). Fig. 3(b) demonstrates that mersalyl causes the retention of 69% of the P_i formed within the mitochondrial matrix. Thus, even when both known P_i carriers are apparently inhibited, a substantial portion of the P_i efflux continues. Other concentrations of mersalyl were tested (i.e. $40\text{nmol}/\text{mg}$ of protein and $120\text{nmol}/\text{mg}$ of protein) and resulted in a less effective inhibition of the P_i efflux pathway (results not shown). Upon pre-incubation with both NEM and mersalyl together (Fig. 4a), an inhibition results that resembles that observed with mersalyl alone (Fig. 3b). The presence of NEM and n-butylmalonate (an inhibitor of the $P_i/\text{dicarboxylate}$ carrier) causes little additional inhibition of P_i efflux over that observed with NEM alone (Figs. 4b and 3a). Finally, Fig. 5 indicates that *p*-CMB, an inhibitor of influx on both known P_i carriers (Fonyó & Bessman, 1968; Coty & Pedersen, 1975) inhibits phosphate efflux by only 23%.

In summary, the standard inhibitors of P_i influx do not inhibit a substantial portion of the P_i efflux

Fig. 2. The effect of oligomycin on P_i production and distribution during valinomycin/ K^+ -stimulated ATP hydrolysis. Reactions were performed as described in the Experimental procedures section. (b) depicts results from incubations that contained $10\mu\text{g}$ of oligomycin/ mg of protein before the mitochondrial addition. (a) shows results for experiments without oligomycin. Data are means from at least three separate mitochondrial preparations. $\square$, Intramitochondrial P_i ; Δ , extramitochondrial P_i ; $\circ$, calculated total P_i .

Fig. 3. The effect of NEM and mersalyl on intramitochondrial and extramitochondrial P_i appearance during valinomycin/K⁺-stimulated ATP hydrolysis

Experimental conditions were as described for Fig. 2(a) except before the mitochondrial addition incubations also contained: (a), 40 nmol of NEM/mg of protein; (b), 80 nmol of mersalyl/mg of protein. □, Intramitochondrial P_i; △, extramitochondrial P_i; ○, calculated total P_i.

Fig. 4. The combined influence of several P_i influx inhibitors on P_i distribution during valinomycin/K⁺-induced ATP hydrolysis

Conditions were as described for Fig. 2(a) except that before the mitochondrial addition incubations also contained: (a) 40 nmol of NEM/mg of protein + 80 nmol of mersalyl/mg of protein; (b), 40 nmol of NEM/mg of protein + 2 mM n-butylmalonate. □, Intramitochondrial P_i; △, extramitochondrial P_i; ○, calculated total P_i.

pathway when the transportable substrate is P_i generated from valinomycin/K⁺-induced ATP hydrolysis.

Characteristics of the mersalyl-insensitive P_i efflux process

Experiments were conducted in an attempt to determine whether the observed mersalyl-insensitive P_i efflux (Fig. 3b) was carrier-mediated or was simply passive diffusion. However, it should be

noted that in the absence of a specific chemical inhibitor of a transport process, it is difficult to obtain evidence that conclusively proves a carrier-mediated mechanism. Fig. 6 depicts the time-dependence and the (*v*) versus [*S*] profile of the mersalyl-insensitive P_i efflux process. In these incubations, a high level of internal phosphate was generated via the valinomycin/K⁺-induced ATP hydrolysis reaction, which was allowed to proceed for 170 s. An inhibitor mix was then added, which

Fig. 5. The ability of *p*-CMB to affect P_i distribution during valinomycin/ K^+ -induced ATP hydrolysis. Incubations were as described for Fig. 2(a) except that 20 nmol of *p*-CMB/mg of protein was included. □, Intramitochondrial P_i ; △, extramitochondrial P_i ; ○, calculated total P_i .

consisted of 4 μ g of oligomycin/mg of protein/100 μ M-DAPP/1 mM-EDTA/Buffer B and, where indicated, 3 nmol of CAT/mg of protein. The efflux of internal phosphate as a function of time was then measured. Fig. 6(a), which is based on data obtained from five separate experiments, demonstrates that CAT had almost no effect on the observed P_i efflux. A rate of efflux and an average internal phosphate level were calculated from each time interval in Fig. 6(a) (after the inhibitor addition) and are presented in a Michaelis–Menten plot in Fig. 6(b). This Figure clearly shows that in the absence of CAT, the P_i efflux process is saturable. These results are consistent with either a high- K_m carrier-mediated process (apparent K_m 65 nmol of P_i /mg of protein; approx. 65 mM, assuming 1 μ l of matrix volume/mg of protein) or alternatively with substrate crowding and subsequent passive diffusion through a possibly non-specific inner-membrane pore (Zierler, 1961; Naftalin, 1970).

Swelling studies were conducted employing incubation conditions identical with those in Fig. 6 (results not shown) and indicate that mitochondria swell during and subsequent to the extensive ATP hydrolysis reaction.

For this reason studies were conducted to determine whether mitochondria were leaky to low-molecular-weight anions (i.e. malate and 2-oxoglutarate) during the incubations described in Fig. 6. Accordingly the latency of the matrix

enzymes fumarase and glutamate dehydrogenase was determined at different times during the efflux reaction. For both enzymes the latency index (i.e. specific activity in the presence of Lubrol/specific activity in the absence of Lubrol) did not change with increasing time in the efflux mixture. The mean latency index values for fumarase and glutamate dehydrogenase were 6.1 and 3.4 respectively. Employing freshly isolated mitochondria values of 2.0 and 5.6 were obtained. Thus, it appears that even though mitochondria are swollen after the extensive ATP hydrolysis reaction, they still retain considerable impermeability to low-molecular-weight anions, as is characteristic of freshly isolated intact mitochondria.

Discussion

The present study sought to characterize mechanism(s) by which P_i within the mitochondrial matrix effluxes through the inner membrane. Specifically, this investigation attempted to determine whether P_i efflux occurs via an atractyloside-sensitive transporter, by the classical operation of the P_i/H^+ and P_i /dicarboxylate carriers and/or by additional processes. We first considered the suggestion that P_i may efflux on an ATP/ADP,0.5 P_i carrier (Reynafarje & Lehninger, 1978).

Experiments presented here show that in the presence of oligomycin, a low-level ATP hydrolysis reaction is catalysed by rat liver mitochondria (Fig. 1). A portion of the P_i is retained within the mitochondrial matrix, whereas the remaining P_i effluxes into the extramitochondrial volume via an unknown mechanism. The addition of atractyloside (or CAT) causes a reduction in both the hydrolysis reaction and in the appearance of P_i in the intramitochondrial and extramitochondrial volumes. In a similar experiment other investigators (Reynafarje & Lehninger, 1978) did not measure total P_i but instead assumed that in the presence of oligomycin, ATP hydrolysis is completely inhibited. Consequently, the observed atractyloside inhibition of extramitochondrial P_i appearance (and ATP disappearance) was thought to be due to an atractyloside-sensitive ATP/ADP,0.5 P_i carrier.

The present findings (i.e. the atractyloside inhibition of ATP hydrolysis), and the data of others (Reynafarje & Lehninger, 1978) seem best explained by the well-documented atractyloside inhibition of the ATP/ADP carrier (Pfaff *et al.*, 1965; Winkler *et al.*, 1968; Vignais *et al.*, 1973). Thus, atractyloside (or CAT) blocks ATP influx through the inner membrane via the ATP/ADP carrier and therefore prevents ATP from being hydrolysed by the F_1 -ATPase. Consequently, a decrease in the appearance of P_i in the intramitochondrial and the extramitochondrial volumes results. Additionally, the results presented here directly show that after the

Fig. 6. The effect of CAT on the efflux of intramitochondrial P_i.

Incubations were performed in which 3 nmol of CAT/mg of protein was either present (●) or absent (○) from the inhibitor mixture. The addition of ATP was considered to be the zero time point. Each datum point in (a) represents the mean of five separate experiments. After the addition of the inhibitor mixture, mean v and $[S]$ values were calculated from each time interval (a) and are plotted in (b). Other conditions were as described in the Experimental procedures section.

generation of high levels of matrix P_i, a mersalyl-insensitive P_i efflux occurs that is not inhibited by CAT (Fig. 6). In summary we found no evidence that supports the existence of an atractyloside (or CAT)-sensitive ATP/ADP,0.5P_i transporter.

In the second part of this study we considered the possibility that P_i may efflux on known P_i transport systems, i.e., either the P_i/H⁺ or P_i/dicarboxylate carriers, or by other mechanism(s). Therefore, the ability of mersalyl, NEM plus *n*-butylmalonate or *p*-CMB to inhibit the efflux of the matrix P_i generated via valinomycin/K⁺-induced ATP hydrolysis was determined. Other workers have shown that these inhibitors almost totally abolish P_i influx on the P_i/H⁺ and the P_i/dicarboxylate carriers (Meyer & Tager, 1969; Tyler, 1969; Guérin *et al.*, 1970; Meijer *et al.*, 1970; Coty & Pedersen, 1974, 1975; Siliprandi *et al.*, 1975). Nonetheless, the present study shows that regardless of the inhibitors employed a substantial portion of P_i efflux continues (Figs. 2–5). For example, it was found that in the presence of *p*-CMB (Fig. 5), approx. 80% of P_i efflux continues. Also, with either mersalyl or NEM significant P_i efflux is still evident. The addition of NEM plus *n*-butylmalonate (Fig. 4b) causes little inhibition above that observed with NEM alone. Finally, incubation with NEM plus mersalyl (Fig. 4a) causes no further inhibition than was observed with mersalyl alone.

Consistent with these observations other investigators have shown that the ability of thiol-specific reagents to inhibit P_i efflux is limited and depends on the incubation conditions employed (Siliprandi *et al.*, 1975; Fonyó & Vignais, 1979; Tyler, 1980). For example, Siliprandi *et al.* (1975) found that during uncoupler-stimulated ATP hydrolysis, mersalyl inhibited P_i efflux by approx. 50%. Upon the addition

of Mg²⁺, the observed inhibition was almost completely abolished. Tyler (1980) showed that, whereas mersalyl substantially inhibited P_i efflux in the presence of antimycin and rotenone (78%), the omission of these electron-transport inhibitors decreased the inhibition to only 14%. Employing the latter conditions, NEM inhibited only about one-half of the observed P_i efflux. Finally, Fonyó & Vignais (1979) reported that during valinomycin/K⁺-induced ATP hydrolysis, mersalyl inhibited efflux nearly completely.

In summary, results presented here and the observations of other investigators (Siliprandi *et al.*, 1975; Reynafarje & Lehninger, 1978; Tyler, 1980) indicate that matrix P_i effluxes through the inner membrane via at least two general pathways. A substantial portion of the efflux occurs via a thiol-inhibitor-sensitive pathway. Our finding that non-penetrant inhibitors (Gaudemer & Latruffe, 1975; Fonyó, 1979) [i.e. mersalyl (Fig. 3b), *p*-CMB (Fig. 5)] prevent this efflux suggests a mechanism involving a protein carrier that contains at least one thiol group that is both essential for activity and is exposed to the outer surface of the inner membrane during a portion of the efflux process. Also since *n*-butylmalonate plus NEM causes little further inhibition over that observed with NEM alone (Figs. 3a and 4b) we conclude that these results are generally consistent with P_i efflux via the classical P_i/H⁺ carrier (Coty & Pedersen, 1975). However, it is noteworthy that this observed P_i efflux pathway maintains a different sensitivity to thiol-specific reagents than does the P_i influx pathway. Perhaps depending upon the incubation conditions employed the P_i/H⁺ carrier assumes differing conformations and/or locations within the inner membrane (Guérin *et al.*, 1970; Papa *et al.*,

1973; Quoc *et al.*, 1977; Fonyó & Vignais, 1980), which then alter the accessibility of its thiol groups.

The other portion of P_i efflux occurs via a thiol-inhibitor-insensitive pathway. The question arises as to whether this portion of the observed P_i efflux is in fact carrier-mediated or is instead passive diffusion. Fig. 6(b) demonstrates that the mersalyl-insensitive P_i efflux is a saturable process, which would therefore tend to suggest it is carrier-mediated. However, Zierler (1961) has shown that the movement of a particle through a non-specific aperture can also display saturation kinetics. Studies measuring the latency of the matrix marker enzymes fumarase and glutamate dehydrogenase indicate that the mitochondrial inner membrane retains considerable impermeability to low-molecular-weight anions (i.e. malate and 2-oxoglutarate) during the mersalyl-insensitive P_i efflux incubations. Thus, the inner membrane does not become non-specifically leaky during the transport reactions. Although it is rather difficult to conclusively prove that a transport process is carrier-mediated in the absence of a specific inhibitor, we suggest that the evidence obtained is more consistent with a carrier-mediated mechanism than with passive diffusion. It is possible that the inhibitor-insensitive P_i efflux pathway may correspond to a fraction of P_i/H^+ carrier molecules in a conformation unreactive toward thiol-specific reagents. Alternatively, a separate P_i efflux carrier may exist (Wehrle *et al.*, 1978; Wehrle & Pedersen, 1981). Further experiments are required to distinguish more clearly the P_i influx and efflux pathways at the molecular level.

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