Identification of the *N*-Ethylmaleimide Reactive Protein of the Mitochondrial Phosphate Transporter†

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ABSTRACT: The mitochondrial phosphate carrier is inhibited by the SH reagents *p*-(hydroxymercuri)benzoate and *N*-ethylmaleimide. Based on an analysis utilizing dodecyl sulfate–polyacrylamide gels, an SH-containing 32 000-dalton protein has been identified as a component of the phosphate carrier system. Two other *N*-[^{3}H]ethylmaleimide-labeled proteins of the inner mitochondrial membrane have been eliminated from this role [Wohlrab, H., & Greaney, J., Jr. (1978) *Biochim. Biophys. Acta 503*, 425] on the basis that band IV (45 000 daltons) is absent from heart sonic submitochondrial particles and band VII (6 500 daltons) does not react with *p*-(hydroxymercuri)benzoate. The mobility of the 32 000-dalton protein (0.43) is lower than that of the γ subunit of the mitochondrial ATPase (0.46) and the carboxyatractyloside binding protein (0.48) on 12.5% dodecyl sulfate–polyacrylamide gels. In these flight muscle mitochondria, 0.87 nmol of *N*-[^{3}H]ethylmaleimide per nmol of cytochrome *a* is bound to the 32,000-dalton protein.

Transport of inorganic phosphate across the inner mitochondrial membrane is required for steady-state phosphorylation of extramitochondrial ADP by oxidative phosphorylation. The phosphate transport occurs as phosphate–hydroxyl anion exchange or as phosphate–proton cotransport (Chappell & Crofts, 1965). This transport is sensitive to sulfhydryl group reactive reagents such as *N*-ethylmaleimide and *p*-(hydroxymercuri)benzoate (Meijer et al., 1970). Isolation of the membrane protein responsible for this transport is necessary in order to completely understand its catalytic mechanism. Several different approaches have been used in attempts to identify the protein(s) responsible for the transport. The difficulty with radioactively labeled SH inhibitors is that they are not specific for the phosphate carrier protein (Coty & Pedersen, 1975; Briand et al., 1976; Hadvary & Kadenbach, 1976; Touraille et al., 1977). Kadenbach & Hadvary (1973) and Guerin & Napias (1978) identified proteins that partition inorganic phosphate into chloroform. Since this transfer of inorganic phosphate is sensitive to SH reagents, they suggested that these proteins might be phosphate carrier proteins. Banerjee et al. (1977) have purified protein(s) from beef heart mitochondria that catalyzes phosphate transport into liposomes in an SH reagent sensitive manner.

We have utilized high-resolution dodecyl sulfate–polyacrylamide gels of *N*-[^{3}H]ethylmaleimide-labeled mitochondria and sonic submitochondrial particles to identify those inner membrane proteins that could be part of the phosphate carrier (Wohlrab & Greaney, 1978; Wolrab, 1978). I present evidence now that only the 32 000-dalton protein provides the SH groups that permit inhibitors to block the transport.

Experimental Procedure

Mitochondria were prepared from the thoraces of 8–12-day-old flies (*Sarcophaga bullata*) in the absence of protease and bovine serum albumin (Wohlrab & Greaney, 1978). Rat heart mitochondria were prepared from retired Sprague–Dawley breeders. After decapitation of the rat, the heart was placed immediately into 0–4 °C isolation medium (0.07 M sucrose, 0.22 M mannitol, 10 mM KMops, and 0.2 mM EGTA, pH 7.2).[1] It was cut up with scissors into very small pieces, and during this process the tissue was rinsed about 3 times with cold isolation medium. The cut tissue was suspended in 10 mL of isolation medium that contained 50 μg of nagarse per mL. The tissue was homogenized with a glass–Teflon homogenizer until 2 min after the initial suspension in the nagarse isolation medium. The homogenate was immediately diluted with 5 volumes of isolation medium and centrifuged at 750*g* for 5 min. The supernatant was filtered through two layers of cheesecloth and centrifuged at 6000*g* for 8 min. The mitochondrial pellet was suspended in 30 mL of isolation medium and centrifuged at 6000*g* for 8 min. The mitochondrial pellet was suspended in 30 mL of isolation medium and centrifuged at 6000*g* for 8 min. The final pellet was suspended in 100 μL of isolation medium and assayed. Passive swelling of the mitochondria was carried out at 22 °C in 100 mM ammonium phosphate, 5 mM ammonium Mops, and 1 mM EGTA at pH 7.2 after preincubating the mitochondria for 2 min at 0–2 °C with inhibitors (Wohlrab & Greaney, 1978). The *N*-[^{3}H]ethylmaleimide-labeling reaction was stopped with dithiothreitol (Wohlrab, 1978). The mitochondria were centrifuged for 2 min at 4 °C in an Eppendorf centrifuge. The pellet was suspended in 0.25 M sucrose and 3.2 mM dithiothreitol and immediately mixed into the dodecyl sulfate sample buffer which contained 1.6 mM dithiothreitol. One minute later, at room temperature, the proteins were alkylated with *N*-ethylmaleimide at a final concentration of 5.5 mM. The dodecyl sulfate–polyacrylamide gel electrophoresis was carried out essentially as described by Laemmli (1970). Gel slices were digested at 65 °C according to Malim & Lofberg (1966). Radioactive gels were generally frozen in liquid nitrogen immediately after termination of the electrophoresis and stored at −10 °C. Cytochrome *a* was determined with an Aminco-Chance spectrophotometer (Wohlrab, 1974). Cytochrome *b* was determined in a similar manner, except $\epsilon_{cm^{-1}}^{mM} = 134$ was used at 430–410 nm for the difference in optical density of aerobic and uncoupled mitochondria without added substrates vs. aerobic, uncoupled, and antimycin A inhibited mitochondria with succinate added. Sonic submitochondrial particles with very high energy transducing efficiency were prepared as published (Wohlrab & Greaney, 1978) from flight muscle mitochondria. The same procedure was used for the preparation of sonic submitochondrial particles from rat heart mitochondria. Fluorography

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[1] Abbreviations used: Mops, 3-(*N*-morpholino)propanesulfonic acid; BPB, bromophenol blue; EGTA, ethylene glycol bis(*β*-aminoethyl ether)-*N*,*N*′-tetraacetic acid.

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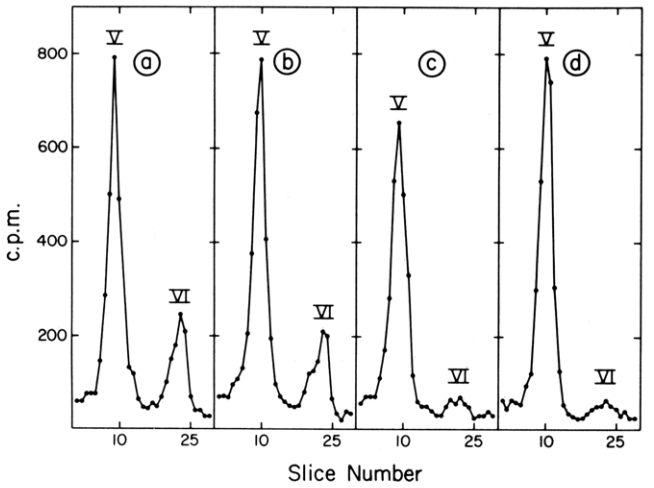

FIGURE 1: Inhibition of *N*-[^{3}H]ethylmaleimide labeling of band VI by carboxyatractyloside. Flight muscle mitochondria were labeled wth 20 nmol of *N*-[^{3}H]ethylmaleimide per nmol of cytochrome *a* and electrophoresed on dodecyl sulfate–polyacrylamide gels. The regions of the gels containing bands V and VI were sliced, digested, and counted. The mitochondrial suspension contained 11.3 μM cytochrome *a* in Mops medium (100 mM KMops and 0.2 mM EGTA, pH 7.2). *N*-[^{3}H]Ethylmaleimide was added to 222 μM (a); 50 μM ADP was added before *N*-[^{3}H]ethylmaleimide (b); 200 μM carboxyatractyloside was added before *N*-[^{3}H]ethylmaleimide (c); the ADP was added after carboxyatractyloside but before *N*-[^{3}H]ethylmaleimide (d).

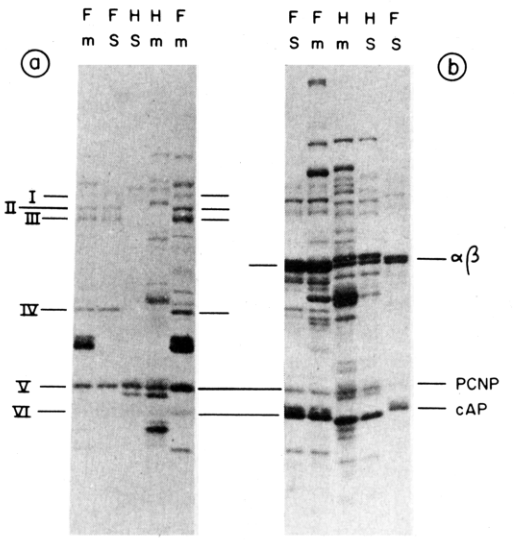

FIGURE 2: Dodecyl sulfate gradient polyacrylamide slab gel of *N*-[^{3}H]ethylmaleimide-labeled mitochondria (m) and sonic submitochondrial particles (s) from flight (F) and heart muscles (H). The slab gel was prepared and run on a Hoefer Model SE-520 electrophoresis apparatus with a 10–25% acrylamide gradient. Only that part of the gel with the higher molecular weight proteins is shown. The slab gel was divided into two parts: (a) is the fluorograph of half of the gel and (b) is the Coomassie Brilliant Blue stains of the other half. The amount of heart mitochondrial protein is expressed as picomoles of cytochrome *b* and that of flight muscle mitochondria as picomoles of cytochrome *a*. The amount of sample applied to the gel from left to right: (a) 8.5, 8.5, 17.5, 17.5, 17.5; (b) 8.5, 8.5, 8.5, 8.5, 3.5. The mitochondria had been labeled with 20 nmol of *N*-[^{3}H]ethylmaleimide per nmol of cytochrome *a* (or cytochrome *b* for heart), and sonic submitochondrial particles were prepared from them. I–VI are the *N*-[^{3}H]ethylmaleimide-labeled proteins of the inner membrane of flight muscle mitochondria; α and β are the subunits of the mitochondrial ATPase; PCNP (band V) is the phosphate carrier *N*-ethylmaleimide binding protein; cAP (band VI) is the carboxyatractyloside binding protein.

of the dodecyl sulfate–polyacrylamide slab gels was carried out according to Bonner & Laskey (1974).

Purified, low molecular weight protein markers were obtained from Gallard-Schlesinger (BDH No. 44247), and *N*-[^{3}H]ethylmaleimide was from New England Nuclear. Its concentration was determined spectrophotometrically (Riordan & Vallee, 1967), and its specific activity agreed within 10% of that specified by the manufacturer. *p*-(Hydroxymercuri)benzoate was obtained from Sigma, and its concentration was determined spectrophotometrically (Boyer, 1954). Dodecyl sulfate was obtained from Gallard-Schlesinger (BDH, specially pure, No. 30176), acrylamide (electrophoresis grade) was from Eastman, and nagarse was from Enzyme Development Corporation, New York, N.Y.

Results

Modified Mobilities of Membrane Proteins. We modified our earlier method (Wohlrab & Greaney, 1978) for the electrophoresis of the mitochondrial membrane proteins in order to increase the separation between the labeled and also the stained proteins. We noticed that switching from Pierce Sequanal grade dodecyl sulfate (0.04%) to BDH (specially pure) (0.1%) dodecyl sulfate changed the mobility of some membrane proteins and yielded much darker Coomassie Brilliant Blue stained protein bands.

The difference in mobility of bands V and VI (Wohlrab & Greaney, 1978) was, in fact, increased 1.65-fold by using 0.1% dodecyl sulfate in the gels and in the running buffer, by changing the composition (Pierce to BDH) of dodecyl sulfate, and by alkylating the dodecyl sulfate solubilized and dithiothreitol-reduced membrane proteins. These modifications caused the band V protein to be well removed from the very intensely stained, about 30 000-dalton proteins among which band VI lies. Klingenberg et al. (1978) recently discussed some of the proteins that might be present in this intensely stained band.

Identification of Band VI with the Carboxyactractyloside Binding Protein. Recently we were able to differentiate between bands V and VI on the basis that the binding of *N*-[^{3}H]ethylmaleimide to band VI continues to increase beyond the concentration that is required to maximally inhibit the phosphate transport (Wohlrab, 1978). Now, in addition, Figure 1 shows that the labeling of protein VI by *N*-[^{3}H]-ethylmaleimide is completely prevented by the prior incubation of the mitochondria with carboxyactractyloside. The labeling of protein V and the other membrane proteins is not affected by carboxyatractyloside.

The amount of *N*-[^{3}H]ethylmaleimide label incorporated into band VI was independent of added ADP. Similarly, the carboxyatractyloside effect was independent of ADP (see Figure 1).

Absence of Band IV from Heart Sonic Submitochondrial Particles. Previous experiments had been designed to eliminate protein band IV or V as the *N*-ethylmaleimide reactive phosphate carrier protein. Both bands are labeled to the same extent in our titration experiments (Wohlrab, 1978). Dramatic functional differences exist between mitochondria from flight muscle and from heart. Since both types of mitochondria do possess a phosphate carrier, they both should possess a band V protein and heart mitochondria might lack the band IV protein.

We carried out swelling experiments and determined that 25 nmol of *N*-ethylmaleimide per nmol of cytochrome *b* is required for maximal inhibition of the phosphate transport in heart mitochondria. We labeled the heart mitochondria with 20 nmol of *N*-[^{3}H]ethylmaleimide per nmol of cytochrome *b* and prepared sonic submitochondrial particles. Figure 2 shows a Coomassie Brilliant Blue stained dodecyl sulfate–polyacrylamide gradient slab gel and a fluorograph of half of the

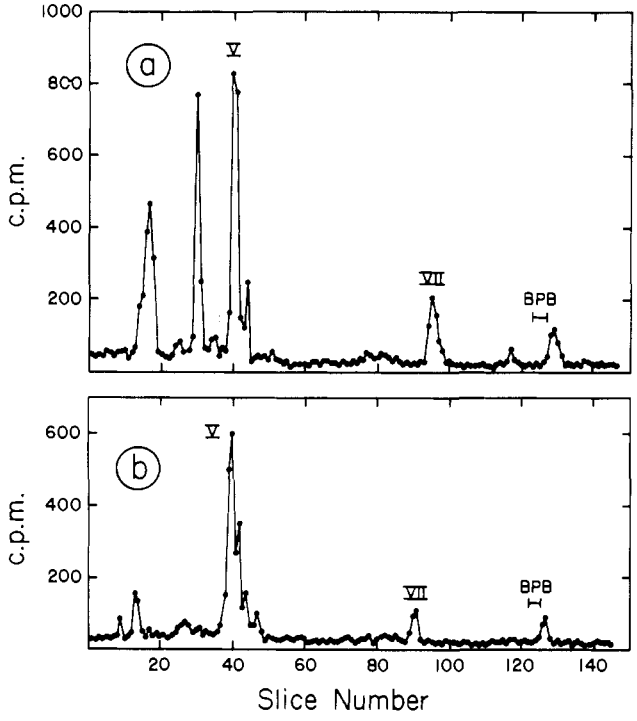

FIGURE 3: Dodecyl sulfate–polyacrylamide gels of sonic submitochondrial particles from flight (a) and heart muscle (b). The acrylamide concentration was increased to 15% to move band VII further away from the tracking dye. The sonic submitochondrial particles were prepared from the *N*-[^{3}H]ethylmaleimide-labeled mitochondria. BPB is the tracking dye bromophenol blue.

gel. The fluorograph demonstrates quite clearly that the sonic submitochondrial particles from heart mitochondria do not possess a band IV protein but do possess a band V protein.

Low Molecular Weight N-Ethylmaleimide Reactive Protein of Sonic Submitochondrial Particles from Flight Muscle and Heart. When we first analyzed the *N*-[^{3}H]ethylmaleimide-labeled mitochondria on dodecyl sulfate–polyacrylamide gels (Wohlrab & Greaney, 1978), we found a low molecular weight protein. At the time we ignored it, since its concentration was very low and Coty & Pedersen (1975) had not detected such a protein in their experiments with the fractionated liver mitochondria. Guerin & Napias (1978) recently reported the isolation of a 10000-dalton protein that is able to partition inorganic phosphate into chloroform in an *N*-ethylmaleimide sensitive manner. They suggested that this protein could be part of the phosphate carrier or the mitochondrial ATPase.

Figure 3 shows that a low molecular weight protein that is labeled by *N*-[^{3}H]ethylmaleimide is present in sonic submitochondrial particles from flight and heart muscle mitochondria. The mobility of the protein from flight muscle is greater by about 5% than that of heart mitochondria on these dodecyl sulfate–15% polyacrylamide gels. This could well be due to species differences since Figure 2 clearly shows that the two ATPase subunits (α and β) as well as the carboxyatractyloside binding protein of the heart and the flight muscle mitochondria differ also to a small extent in their mobilities. The low molecular weight protein, on the basis of this comparison, could thus well be part of the phosphate carrier.

Effect of p-(Hydroxymercuri)benzoate on the Labeling of Membrane Proteins by N-[^{3}H]Ethylmaleimide. *p*-(Hydroxymercuri)benzoate inhibits the passive swelling of mitochondria in ammonium phosphate (Figure 4). The amount of *p*-(hydroxymercuri)benzoate required per cytochrome *a* is about the same as that of *N*-ethylmaleimide (Wohlrab, 1978). The inner mitochondrial membrane is essentially impermeable

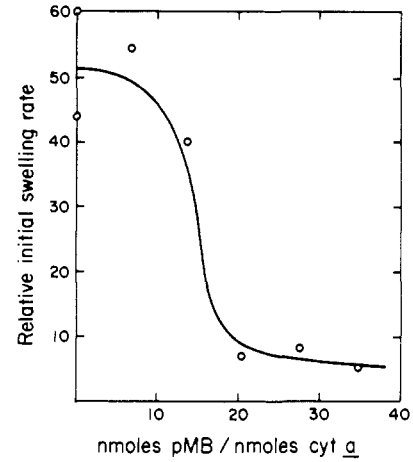

FIGURE 4: The inhibition of passive swelling of flight muscle mitochondria by *p*-(hydroxymercuri)benzoate. The experiments were carried out as described under Experimental Procedure [see also Wohlrab & Greaney (1978)].

to *p*-(hydroxymercuri)benzoate (Gaudemer & Latruffe, 1975). One would expect then that *p*-hydroxymercuribenzoate reacts with fewer SH groups than *N*-ethylmaleimide. We would expect also that only those SH groups that react both with *p*-(hydroxymercuri)benzoate and *N*-ethylmaleimide would qualify as the phosphate carrier *N*-ethylmaleimide reactive protein.

We exposed mitochondria to 27 nmol of *p*-(hydroxymercuri)benzoate per nmol of cytochrome *a* to inhibit the phosphate transport. We then added 27 nmol of *N*-[^{3}H]-ethylmaleimide per nmol of cytochrome *a*. Figure 5 shows that the *N*-[^{3}H]ethylmaleimide label of the low molecular weight protein is not affected by the previous exposure to *p*-(hydroxymercuri)benzoate. It should be noted that peak V, on the other hand, is not labeled by *N*-[^{3}H]ethylmaleimide in the *p*-(hydroxymercuri)benzoate-exposed mitochondria. Figure 6 is of gels with the peak VI protein (carboxyatractyloside binding protein) near the bottom of the gel; i.e., the low molecular weight proteins have been run off the gels. Of the labeled inner membrane proteins (Wohlrab & Greaney, 1978), only peaks II, IV, and V are affected by the *p*-(hydroxymercuri)benzoate prelabeling procedure.

Discussion

Two basically different approaches have been used in the past to isolate and characterize intrinsic membrane transport proteins: (a) the intact membranes are fractionated and the fractions are individually incorporated into lipid vesicles and transport is assayed or (b) a radioactive probe (an inhibitor in most cases) that is known to react with the carrier is used as a marker to isolate protein(s) that can be used—in the absence of the probe—in reconstitution studies. We are using the latter approach and have identified the protein of the mitochondrial phosphate carrier that possesses the *N*-ethylmaleimide reactive SH groups.

The identification depends upon two assumptions: (1) that sonic submitochondrial particles possess the phosphate carrier and (2) that *p*-(hydroxymercuri)benzoate and *N*-ethylmaleimide react with the same SH group on the phosphate carrier. Rhodin & Racker (1974) and Zoccarato et al. (1977) demonstrated that sonic submitochondrial particles from heart do possess SH reagent sensitive phosphate transport when the particles are loaded with $MnCl_2$ or $CaCl_2$ as phosphate sinks. The question as to whether *p*-(hydroxymercuri)benzoate and *N*-ethylmaleimide react with the same SH groups on the phosphate carrier remains strictly unanswered. It is known

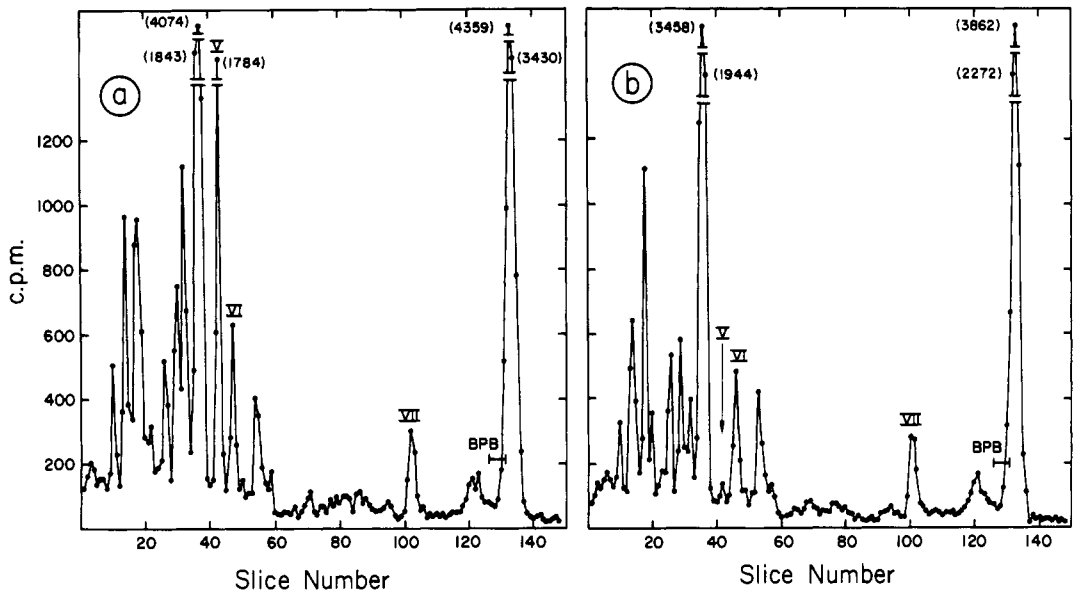

FIGURE 5: Dodecyl sulfate–polyacrylamide gels of flight muscle mitochondria labeled with *N*-[^{3}H]ethylmaleimide in the absence (a) and presence (b) of *p*-(hydroxymercuri)benzoate (27 nmol/nmol of cytochrome *a*). The mitochondria (11 μM cytochrome *a*) were labeled with 280 μM *N*-[^{3}H]ethylmaleimide. The gels contained 15% acrylamide. BPB is the tracking dye bromophenol blue.

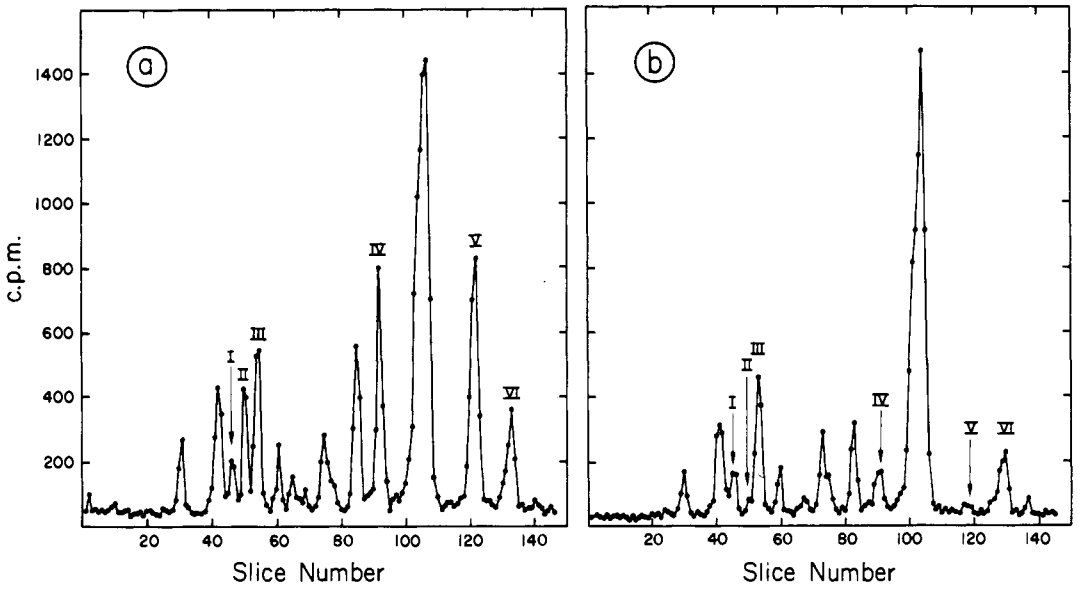

FIGURE 6: Dodecyl sulfate–polyacrylamide gels of flight muscle mitochondria labeled with *N*-[^{3}H]ethylmaleimide in the absence (a) and the presence (b) of *p*-(hydroxymercuri)benzoate (27 nmol/nmol of cytochrome *a*). Polyacrylamide gels with 12.5% acrylamide were used, and the low molecular weight proteins were run off.

that *p*-(hydroxymercuri)benzoate has access to SH groups of the dicarboxylate carrier that are inaccessible to *N*-ethylmaleimide (Meijer et al., 1970). Our experiments, on the other hand, clearly show that *N*-ethylmaleimide has access to SH groups that are inaccessible to *p*-(hydroxymercuri)benzoate. The indirect evidence that suggests that the same SH groups of the phosphate carrier are accessible to *p*-(hydroxymercuri)benzoate and *N*-ethylmaleimide comes from experiments of the type carried out by Coty & Pedersen (1975) who inhibited the phosphate transport with *p*-(hydroxymercuri)benzoate, added excess *N*-ethylmaleimide, and then reversed the inhibition with dithiothreitol. The reactivated phosphate transport is sensitive to *N*-ethylmaleimide.

The sonic submitochondrial particles that were prepared from *N*-[^{3}H]ethylmaleimide-labeled flight muscle mitochondria possess seven labeled proteins (Wohlrab & Greaney, 1978). While we already demonstrated that band VI could not be the phosphate carrier *N*-ethylmaleimide binding protein (Wohlrab, 1978), we have now presented evidence that band VI is the carboxyatractyloside binding protein. *N*-Ethylmaleimide reactive protein(s) of mitochondria that had been implicated in phosphate transport (Coty & Pedersen, 1975; Briand et al., 1976; Hadvary & Kadenbach, 1976) is known to have a molecular weight close to that of the carboxyatractyloside binding protein, which has a molecular weight of 30000 (Klingenberg et al., 1978). Touraille et al. (1977) were not able to decide on the basis of dodecyl sulfate–polyacrylamide gels whether the *N*-ethylmaleimide reactive phosphate transport protein is different from the carboxyatractyloside binding protein. Shertzer et al. (1977), on the other hand, demonstrated in reconstitution studies that different heart mitochondrial membrane fractions stimulate ADP–ATP exchange and phosphate transport in lipid vesicles. We now have clearly separated two *N*-ethylmaleimide reactive proteins that are intrinsic to the inner mitochondrial membrane, i.e., protein bands V and VI. Our evidence that band

VI is the carboxyatractyloside binding protein and not the *N*-ethylmaleimide sensitive protein of the phosphate carrier is that (a) it is saturated with *N*-ethylmaleimide at much higher inhibitor concentrations than the band V (Wohlrab, 1978), (b) the reaction of *N*-ethylmaleimide with band VI is completely prevented by carboxyatractyloside, and (c) the SH group(s) of band VI is inaccessible to *p*-(hydroxymercuri)-benzoate at the concentration used to inhibit the phosphate transport. The last part of the evidence could have been predicted from experiments by Vignais & Vignais (1972) who demonstrated that only membrane-permeable SH reagents (see Gaudemer & Latruffe, 1975) are effective in inhibiting the adenine nucleotide translocase. Their experiments with *p*-(hydroxymercuri)benzoate were not conclusive, possibly because they used more than 10 times as much inhibitor than we did. While Vignais & Vignais (1972) found that ADP plus *N*-ethylmaleimide reduces the binding of atractyloside to mitochondria, our experiments demonstrate that carboxyatractyloside prevents *N*-ethylmaleimide binding to the carboxyatractyloside binding protein.

Guerin & Napias (1978) recently demonstrated that a 10000-dalton mitochondrial protein is able to catalyze the partitioning of inorganic phosphate into chloroform and that the extraction is reduced in the presence of *N*-ethylmaleimide. They suggested that this proteolipid, which they also could isolate from the oligomycin-sensitive ATPase complex, might be a protein of the phosphate carrier system. Our experiments demonstrate that there is no inner membrane protein of low molecular weight possessing an SH group sensitive to *p*-(hydroxymercuri)benzoate and *N*-ethylmaleimide at concentrations that inhibit the phosphate transport. Our experiments also show that band VII (6500 daltons) is barely detectable in Coomassie Brilliant Blue stained gels (see also Wohlrab & Greaney, 1978) and reacts with only about $^1/_5$ the amount of *N*-[^{3}H]ethylmaleimide that we find to be present in band V (32000 daltons).

Our present results suggest very strongly that the band V protein is the *N*-ethylmaleimide-sensitive protein of the phosphate carrier. We eliminated protein band IV since it is absent from sonic submitochondrial particles from rat heart. We believe that it is unlikely that bands IV and V comigrate in heart mitochondria. Furthermore, preliminary experiments have now shown that the high molecular weight *N*-[^{3}H]-ethylmaleimide reactive protein (about 75000 daltons; see Figure 3b) does not react with *p*-(hydroxymercuri)benzoate. This result supports the titration results (Wohlrab, 1978) which eliminated the band II protein. It is very likely that the band V protein by itself, or possibly as a dimer or oligomer, is responsible for the phosphate transport. We are presently carrying out experiments to test this hypothesis.

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