Natural and Azido Fatty Acids Inhibit Phosphate Transport and Activate Fatty Acid Anion Uniport Mediated by the Mitochondrial Phosphate Carrier*

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The electronotive P$_i$ uptake via the phosphate carrier (PIC) in rat liver and heart mitochondria is inhibited by fatty acids (FAs), by 12-(4-azido-2-nitrophenylamino)dodecanoic acid (AzDA) and laurlybenzoic acid (−1 μM doses) and by lauric, palmitic, or 12-azidododecanoic acids (−0.1 mM doses). In turn, reconstituted E. coli-expressed yeast PIC mediated anionic FA uniport with a similar pattern leading to FA cycling and H$^+$ uniport. The kinetics of P$_i$/P$_i$ exchange on recombinant PIC in the presence of AzDA better corresponded to a competitive inhibition mechanism. Methanephosphonate was identified as a new PIC substrate. Decanephosphonate, butanephosphonate, 4-nitrophenylphosphate, and other P$_i$ analogs were not translocated and did not inhibit P$_i$ transport. However, methylenediphosphonate and iminodi(methylene)phosphonate inhibited both electronotive P$_i$ uptake and FA cycling via PIC. AzDA analog 16-(4-azido-2-nitrophenylamino)-[3H]-hexadecanoic acid (3H-AzHA) bound upon photoactivation to several mitochondrial proteins, including the 30- and 34-kDa bands. The latter was assigned to PIC due to its specific elution pattern on Blue Sepharose and Affi-Gel. 3H-AzHA photolabeling of recombinant PIC was prevented by methanephosphonate and diphosphonates and after premodification with 4-azido-2-nitrophenylphosphate. Hence, the demonstrated PIC interaction with mono- and divalent long-chain FA anions, but with divalent phosphonates of short chain only, indicates a pattern different from that valid for the mitochondrial uncoupling protein-1.

The mitochondrial phosphate carrier (PIC)$^1$ (1–10) belongs to the well established gene family of homologous mitochondrial anion carrier proteins (10–12). It was thought to mediate a stoichiometric P$_i$/H$^+$ symport (7, 13), but alternatively, a P$_i$/OH$^-$ antiport has been proposed to be the most plausible mode (13, 14). In addition to monovalent phosphate (15), arsenate and divalent monofluorophosphate (16) are also translocated. With the human genome available, a major effort will be made in the coming years to ascribe particular phenotypes to the revealed genes. Some proteins possess several functions; consequently, a complete spectrum of functions for a given protein should be known. It is not uncommon that a carrier fulfills several functions. For example, the ADP/ATP carrier is thought to participate in the so called mitochondrial permeability transition (17), which is also activated by fatty acids (18). Although they share a similar trans-membrane folding (10–12), MACPs exert diverse functions and conduct anions of different charge and by different modes (symport, antiport, uniport). They all contain positively charged and membrane-embedded arginines or lysines located on trans-membrane α-helices (11, 12), which may contribute to the putative anion binding sites for fatty acid anions and other hydrophobic anions (12, 19). Interaction of some MACPs with fatty acids seems to represent another common feature, probably their second phenotype. Consequently, probing carriers with artificial hydrophobic substrate analogs might reveal some new structure/function relationships.

Indeed, PIC (20) as well as homologous proteins, the ADP/ATP carrier (AAC; Refs. 19, 21, and 22) and uncoupling proteins UCP1 of brown adipose tissue mitochondria (19, 23–25), plant uncoupling mitochondrial protein (26), and recently discovered UCP2 and UCP3 (27) have been shown to interact with fatty acids (FAs) and are predicted to mediate the uniport of fatty acids (19–23, 26, 27). This allows for the FA uncoupling cycle, suggested by Škulachev (28, 29). For UCPs, the FA cycling should represent a major physiological function, whereas for AAC, PIC, and other carriers, it could be a “tolerated” side function (19, 20). FAs usually inhibit the other functions of the carriers. Thus, anion uniport via UCP1 is inhibited by various FAs (23), including 12-(4-azido-2-nitrophenylamino)dodecanoic acid (AzDA; Ref. 24). The latter also inhibits ADP uptake on AAC (21) and the dicarboxylate carrier-

*N-ethylmaleimide; PUMP, plant uncoupling mitochondrial protein; UCP, uncoupling protein (UCP1), brown adipose tissue-specific UCP; UCP2, ubiquitous UCP; UCP3, predominantly muscle-specific UCP; BMCP and UCP4, brain-specific UCPs; PAGE, polyacrylamide gel electrophoresis; TEA, tetraethylammonium; TES, 2-[(2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]ethanesulfonic acid.

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‡ The abbreviations used are: PIC, mitochondrial phosphate carrier; AAC, ADP/ATP carrier; AzDA, 12-(4-azido-2-nitrophenylamino)dodecanoic acid; $^3$H-AzHA, 16-(4-azido-2-nitrophenylamino)-[3H]hexadecanoic acid; AzNPP, 4-azido-2-nitrophenylphosphonate; BSA, bovine serum albumin; BS, blue Sepharose (Cibacron blue affinity agarose); FA, fatty acid; HTTP, hydroxylation phosphate; LS, light scattering; MDPH, methylene-diphosphonate; MOPS, 3-(N-morpholino)propanesulfonic acid; NEM,
ated transport (30). UCPI was also found as a prominently labeled band among PAGE-separated proteins of brown adipose tissue mitochondria, photolabeled with $^3$H-AzDA (24), or with its hexadecane acid analog ($^3$H-AzHA; Ref. 25). $^3$H-AzHA labeling was successfully applied to AAC as well (21). PIC has also been found to be partially inhibited by fatty acids; however, it has been interpreted in terms of a surface charge effect (31). Besides FAs, the only reported hydrophobic substrate analog was 4-azido-2-nitrophenylphosphate, which inhibited $P$ transport by PIC only upon photoreaction when it was covalently attached to PIC after UV irradiation (32). Hence, the specificity of FA interaction with PIC remains to be elucidated, mainly to determine whether FAs interfere with a putative $H^+\langle OH^+\rangle$ translocation pathway or with a $P_i$ binding site or translocation pathway in the PIC structure. It is not known whether an $H^+\langle OH^+\rangle$ translocation pathway is identical or overlapping with the $P_i$ pathway. A possibility that $H^+$ flux concomitant to $P_i$ flux during the physiological electroneutral $P_i$ flux during the physiological electroneutral

In this paper, we studied both inhibition of PIC by fatty acids and other hydrophobic anions, namely hydrophobic phosphate analogs, as well as fatty acid cycling mediated by PIC. Our results demonstrate that the pattern of PIC interaction with hydrophobic anions is distinct from that of UCPI (33) and other mitochondrial uncoupling proteins.

**EXPERIMENTAL PROCEDURES**

**Materials**—Alkylphosphonates were purchased from Fluka (Switzerland); materials for electrophoresis, hydroxylapatite (Bio-Gel HTP), and Affi-Gel were from Bio-Rad. AzDA and $^3$H-AzHA were synthesized as described elsewhere (24, 25). The autoradiography enhancer ENTRASY NEOF-992 was from PerkinElmer Life Sciences. Other materials were from Sigma.

**Chemical Syntheses**—For 12-azidododecanoic acid, 100 mg of 12-bromododecanoic acid methylster (0.36 mmol) and 116 mg of Na$\text{CN}(1.8$ mmol) were stirred in 2 ml of dry $N_2$-$V$-dimethylformamide at 20°C for 12 h. The reaction mixture was diluted with water and acidified with 5 $\times$ HCl and 12-azidododecanoic acid was extracted several times with ether. The organic layer was dried and evaporated to an oil, which was hydrolyzed by stirring with 0.3 ml of 1 N methanolic KOH (20°C for 12 h) and subsequently diluted with water and acidified with 5 $\times$ HCl, and 12-azidododecanoic acid was extracted several times with ether. The organic fraction was washed with water, dried, and evaporated to a colorless oil (81 mg) that crystallized in a refrigerator. The product exhibited a $^{13}$C NMR spectrum as described for 2′ ENTRASY NEOF-992 from PerkinElmer Life Sciences. Other materials were from Sigma.

**Phosphate Transport in Mitochondria**—Anion transport in mitochondria was indicated by osmotic swelling while detecting light scattering (LS) at 530–550 nm as an apparent absorbance on a diode-array spectrophotometer (Spectronics 3000). LS intensity, reflecting the inverse volume, allows measurement of a protein concentration as low as 0.2 mg/ml in an optimum 40% isotonic medium osmolarity (270 mosmol as calculated using a spectrophotometric method). The known external $P_i$ (the total $P_i$ amount in the sample) and the protein amount in the sample in mg (usually 4 mg) were calculated by measuring $[^{33}P]$phosphate flux using a forward exchange procedure (8, 9). Kinetics was evaluated by variations of the external $[P_i]$, while 30 mM $[P_i]$ was kept constant in the vesicle interior. Proteoliposomes prepared in a medium containing 30 mM $[P_i]$ were washed on a Sephadex G25–300 soaked with the desired external $[P_i]$, while PIC was blocked by mersalyl (0.3 mM). Transport was then initiated by the addition of spectrin (0.5 mM). An inhibitor-stop assay has been employed, so that 64 mM pyridoxal phosphate was added after a given time. The sample was then passed through the Dowex column (1-X10, Cl− form) to remove the external label. Forward exchange rates were determined by fitting the time course of isotope equilibration to a single exponential, $Y = A (l - e^{-kt}) + B$, leading to the first order rate constant $k$ (min$^{-1}$). A specific activity in amol/min·mol of protein was determined by using $b$, the known external $[P_i]$ (the total $P_i$ amount in the sample) and the protein amount in the sample in mg (usually 4 mg) of PIC protein, which equals a lipid/protein ratio of 400 or a molar lipid/PIC monomer ratio of 18,100.

Reconstitution was performed by 14 cycles of detergent removal on a single column as described elsewhere (8, 9). Briefly, 94 $\mu$l of PIC (e.g. 0.596 mg/ml) solubilized from inclusion bodies. 224 $\mu$l of preformed liposomes (224 mg of lipid) from E. coli lecithin (Avanti Polar Inc.), and 140 $\mu$l of Triton X114 were vortexed and supplied by stock solutions to obtain 1.4 ml of suspension containing PIC (e.g. 56 $\mu$g), 30 $\mu$m K$P$, 50 $\mu$m K-HEPES, pH 6.8. It was passed 14 times over a column filled with 0.6 g of Bio-Beads-SM2 (Bio-Rad).

**Proton Uniport Induced by Fatty Acids in Proteoliposomes**—PIC reconstituted with 4-azido-2-nitrophenylphosphate, isolated as 4-azido-2-nitrophenylphosphate, which is in good accordance with its MS

$\beta = (P/P_e)(\alpha - 1)$

(Eq. 1)

where $P$ is protein concentration, ($P_e = 1$ mg/ml), and $\alpha$ is a machine constant, 0.1163 for the Spectronics 3000. $\beta$ was determined by measuring $[^{33}P]$phosphate flux using a forward exchange procedure (8, 9). Kinetics was evaluated by variations of the external $[P_i]$, while 30 mM $[P_i]$ was kept constant in the vesicle interior. Proteoliposomes prepared in a medium containing 30 mM $[P_i]$ were washed on a Sephadex G25–300 soaked with the desired external $[P_i]$, while PIC was blocked by mersalyl (0.3 mM). Transport was then initiated by the addition of spectrin (0.5 mM). An inhibitor-stop assay has been employed, so that 64 mM pyridoxal phosphate was added after a given time. The sample was then passed through the Dowex column (1-X10, Cl− form) to remove the external label. Forward exchange rates were determined by fitting the time course of isotope equilibration to a single exponential, $Y = A (l - e^{-kt}) + B$, leading to the first order rate constant $k$ (min$^{-1}$). A specific activity in amol/min·mol of protein was determined by using $b$, the known external $[P_i]$ (the total $P_i$ amount in the sample) and the protein amount in the sample in mg (usually 4 mg) of PIC protein, which equals a lipid/protein ratio of 400 or a molar lipid/PIC monomer ratio of 18,100.

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$\beta = (P/P_e)(\alpha - 1)$

(Eq. 1)
added to initiate H⁺ efflux. Measured fluorescence traces were converted into “H⁻ traces” (20), from which the derived rates in mmol-1 mg of lipid-1 were multiplied by the internal vesicle volume (estimated from SPQ volume distribution) and divided by a surface of 1 mg of liposomes (in μm²) so that an H⁻ flux density per μm² was obtained in pmol of H⁺ s⁻¹ μm⁻².

**Photoaffinity Labeling**—Mitochondrial photomodifications with AzDA and photoaffinity labeling with H³-AzHA were performed using protocols described for brown adipose tissue mitochondria (25). Rat liver or rat heart mitochondria (3 mg of protein) were resuspended in 10 ml of BSA-free isolation medium and shaken for 10 min in an ice bath. H³-AzHA (nonradioactive AzDA) was added to reach a final concentration of 0.46 μM (1.5 nmol/ml of protein), and the mixture was shaken in an ice bath, first in darkness for 10 min and then for 10 min under UV illumination with a 400-watt xenon arc lamp equipped with a WG 8 filter (Schott Glass, Germany) transmitting light above 270 nm. Labeled mitochondria were washed by three centrifugations with BSA and three without BSA at 8500 × g. The last pellet was resuspended in 100 μl of the sucrose medium.

**Isolation of Rat Heart Phosphate Carrier**—Chromatography on hydroxyapatite was conducted using stepwise fractionation on spin columns. Lower loads of mitochondrial octylpentaoxyethylene or Triton X-100 extract (45 mg of protein per 3 g of detergent) gave higher yields of PIC and AAC content. The labeled mitochondria were either applied to the HTP column, or proteins contained in the HTP pass-through (38) or X-100 extract (45 mg of protein per 3 g of detergent) gave higher yields of PIC, since the combination of BS and Affi-Gel excludes most proteins of the HTP eluate.

Laemml SDS-PAGE was conducted, either on a Mighty Small II apparatus (Hoefer, minigels), or on a Protean IIxi (Bio-Rad, 15-cm gels). Laemmli SDS-PAGE was conducted, either on a Mighty Small II apparatus (Hoefer, minigels), or on a Protean IIxi (Bio-Rad, 15-cm gels).

**RESULTS**

**Inhibition of Phosphate Transport by Natural and Azido Fatty Acids**—Rapid electroneutral phosphate uptake was induced in rat liver (Fig. 1a) or rat heart (Fig. 1b) mitochondria as a passive swelling initiated by nigericin in 44 mM potassium phosphate, pH 7.4. Participation of PIC is indicated by electro neutrality and by the specific inhibitory pattern (1–6) when both NEM and mersaryl are added (Fig. 1a). FAs such as laurate (Fig. 2a) and palmitic acid only inhibited P⁺ uptake in high concentrations. Lauric acid exhibited a Ki of 250 μM (Fig. 2a). On the contrary, a weakly inhibitory action was found with natural FAs, 10 μM AzDA inhibited P⁺ uptake by more than 90% (Fig. 1a), and the estimated Ki was 3.8 μM (Fig. 2a). A substantial inhibitory strength was exhibited by heptylbenzoic acid (Ki of 89 μM; Fig. 2b). 12-Azidododecanoic acid was inhibiting with a lower potency (Ki of 310 μM; Fig. 2b). The protein-independent swelling (induced by nigericin in potassium acetate) was only affected by 10% with 10 μM AzDA, suggesting that AzDA produces no major nonselective side effect on the inner mitochondrial membrane. AzDA, even at 50 μM doses, did not inhibit swelling in sodium acetate, which reflects the electroneutral Na⁺/H⁺ antiporter (Fig. 2a, 2b). No inhibition of the latter by any FA tested was found, nor inhibition of pyruvate carrier (41), thus demonstrating specificity for PIC.

Interestingly, the FA derivatives, which were previously found to be unable to flip-flop across the lipid bilayer, such as 12-hydroxylauric (Fig. 2b), phenylvaleric, and docosanedioic acid, did not affect P⁺ transport. Since the other FAs tested, including 12-azidododecanoic acid, were confirmed to possess the ability of fast flip-flop, we suggest that it is a specific (U-shape) conformation in the membrane (42) that prevents these inactive FAs from interacting with PIC.

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*P. Jezek, unpublished data.*
activated AzDA also inhibited P_i uptake in mitochondria with an apparent IC_50 of 4 μM. Since the mitochondria were first irradiated in the presence of AzDA, washed with BSA, and then reisolated, the apparent IC_50 is not directly comparable. Coincidentally, however, it is the same as the above reported K_i value; IC_50 of 4 μM was obtained when mitochondria preincubated first with AzDA in the dark were washed with BSA and reisolated. Consequently, UV illumination does not seem to strengthen the AzDA inhibition. AzDA binding is apparently so tight that no effective washing can eliminate it.

Kinetics of Fatty Acid Inhibition of Phosphate Transport—To establish the type of inhibition by fatty acids, kinetic measurements with the reconstituted E. coli-expressed yeast PIC have been performed. P_i/P_i exchange was measured as a forward 32P uptake into proteoliposomes containing recombinant PIC, while varying external P_i between 1 and 30 mM. Kinetics of such P_i/P_i exchange determined in the absence or presence of 100 μM AzDA was found to agree better with a competitive mechanism (Fig. 3). Although data cannot definitively distinguish between the competitive and noncompetitive type of kinetics, fits of the competitive model gave better agreement. The derived V_max in control was 0.55 μmol of P_i/min (mg of protein)^{-1}, K_m was 6.5 mM, and derived K_i from the data measured with 100 μM AzDA was 99.5 μM. When the direct plots V versus [P_i] were fitted by nonlinear regression to the Michaelis-Menten equation, the derived V_max values in control and with 100 μM AzDA were 0.74 and 0.77 μmol of P_i/min (mg of protein)^{-1}, respectively, and K_m was 9.3 μM in control. Screening of Possible Hydrophobic Substrates of Mitochondrial Phosphate Carrier—Interaction of PIC with FAs suggested that PIC could also interact with some other amphiphilic anions. Therefore, we evaluated whether hydrophobic phosphate analogs are transported by PIC or act as competitive inhibitors. Contrary to the alkylsulfonate translocation by UCP1 (33), which is faster with the increasing chain length, decanephosphonate and butanephosphonate were not transported at a significant rate by PIC, and neither inhibited P_i transport up to a 0.7 and 100 μM dose, respectively. We found that only phosphonate with the shortest chain, methylphosphonate, is the PIC substrate. Methylphosphonate exhibited the same transport characteristics as P_i transport, including NEM sensitivity (Fig. 4) and inhibition by lauric acid, 12-azidodecanoic acid, and AzDA (Fig. 4).

We also attempted to evaluate whether some other amphiphilic compounds are transported by PIC or inhibit P_i transport in mitochondria. While screening various mono-, di-, and trialkylsulfonates and benzene mono-, di-, and tri sulfonates or anions derived from phosphate and phosphonate, such as phosphoformate, phosphopyruvate, phosphogluconate, and 4-nitrophenylphosphate, we found no such case. Particularly, we confirmed that phosphofumarate does not inhibit the net electroneutral P_i uptake, as reported previously (8). On the contrary, methylenediphosphonate and iminodi(methylene-phosphonate) were found to be strong inhibitors (Fig. 5a and b).
across the lipid bilayer, thus carrying H
interpreted in terms of FA cycling (23, 28), in which uniport of
sensitive to diphosphonates (20). As for AAC and UCPs, it has been
Swelling of rat liver mitochondria in 44 mM potassium methanephos-
Other FAs tested gave similar kinetics (Fig. 6
similar to the one observed in protein-free liposomes (20).
V
12-azidolauric (0.55) acids. Note that FAs that are unable to
butterylbenzoic (1.49), myristic (1.35), lauric (0.96; Fig. 6
Menten kinetics (Fig. 6
to induce H
b
Ty
labeled with3H-AzHA (Fig. 7). This is very similar to the
 again in proteoliposomes with the reconstituted recom-
 Although inhibitory to Pi transport, FAs were previously found
analog, lacking the azido group, 4-nitrophenylphosphate, did
Possible Fatty Acid Cycling Mediated by Phosphate Carrier—
Although inhibitory to Pi transport, FAs were previously found
to induce H
uniport in proteoliposomes containing PIC, sen-
K
values for PIC are much higher than those
To confirm that PIC is the protein interacting with 3H-
photolabeling of brown adipose tissue mitochondria, which
yielded UCPI as the major labeled band (25). Among the la-
beled proteins, the most apparent were 30- and 34-kDa bands
3H-AzHA Labeling of Partially Purified Phosphate Carrier Com-
promising the affinity chromatography steps, selective
to determine the final product as PIC. We solubi-
lized rat heart and liver mitochondria by octylpentaoxyethyl-
ene or Triton X-100, passed the extracts through HTP, and
toPIC. It follows a Michaelis-
Ki
values of 1.5 (AzNPP_i, ○) or 4.9 mM (a, filled hexagons, MDPh) and 5.2 mM (b) and Hill coefficients (n_H) of 1.7, 2.4, and 1.97, respectively, were derived.

FIG. 4. Methanephosphonate uptake in rat liver mitochondria. Swelling of rat liver mitochondria in 44 mM potassium methanephosphonate, 5 mM Tris-MOPS, pH 7.2, containing 2 μM rotenone, 0.25 μM antimycin, and 50 μM atractyloside was induced by 2 μM nigericin (Nig). The effects of 10 μM AzDA and 10 μM mersalyl are illustrated. The +NEM trace was recorded with mitochondria preincubated with 400 μM NEM (2 μmol/mg protein) for 30 s.

The K_i values derived for their inhibition were 4.9 and 5.2 mM, respectively. However, they were not transported by PIC. Also, although 4-azido-2-nitrophenylphosphate (AzNPP_i) was previ-
ously found to inhibit swelling in NH_3-Pi only when photoacti-
ated (32), in our study it inhibited the nigericin-induced Pi
permitted by the use of protein-free liposomes. Moreover, we also
ruled out that the inhibitory effect of FAs on PIC is not due to
an unknown component in the mitochondrial suspension.

Possible Fatty Acid Cycling Mediated by Phosphate Carrier—

Using a rather small amount (1.5 nmol/mg of protein) of highly
tritiated azido-FA (3H-AzHA) incubated with rat heart or rat
liver mitochondria and subsequently illuminated by UV light,
only a small portion of numerous mitochondrial proteins were
labeled with 3H-AzHA (Fig. 7). This is very similar to the
FIG. 5. Dose responses for inhibition of phosphate uptake in rat liver mitochondria by 4-azido-2-nitrophenylphosphate and
methylene(diphosphonate) (a) and iminodi(methylenephospho-
ate) (b). P_i uptake was assayed as described in the legends to Figs. 1
and 2. Theoretical fits (solid lines) to the Hill equation are also shown,
from which the K_i values of 1.5 (AzNPP_i, ○) or 4.9 mM (a, filled hexagons, MDPh) and 5.2 mM (b) and Hill coefficients (n_H) of 1.7, 2.4, and 1.97, respectively, were derived.

3H-AzHA Labeling of Phosphate Carrier in Mitochondria—
Using a rather small amount (1.5 nmol/mg of protein) of highly
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liver mitochondria and subsequently illuminated by UV light,
only a small portion of numerous mitochondrial proteins were
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Fig. 6. Fatty acid-induced H⁺ uniport in proteoliposomes containing PIC. a, H⁺ flux density \( J \) is a function of total lauric acid concentration in the absence (○) and presence of 10 mM methylenediphosphonate (□). The H⁺ efflux induced by various lauric acid concentrations, expressed as flux density in 10⁻⁶ pmol of H⁺ s⁻¹ μm⁻², is plotted versus total lauric acid concentrations. Error bars represent S.D. values of two or three estimations; when omitted, S.D. was the size of the symbol. b, Eadie-Hofstee plot for the differential methylenediphosphonate-sensitive flux calculated from the data of a. The derived \( V_{\text{max}} \) amounted 168 × 10⁻⁶ pmol of H⁺ s⁻¹ μm⁻², which is 0.962 nmol of H⁺ s⁻¹ (mg of lipid)⁻¹. The apparent \( K_m \) was about 278 μM. c, H⁺ flux density \( J \) as a function of total myristic (■), oleic (▲), and 12-hydroxylauroic acid concentration (△). d, H⁺ flux density \( J \) as a function of total heptylbenzoic (filled hexagon) and 12-azidolauroic acid (▼) concentration, in the absence and presence of 10 mM methylenediphosphonate (open symbols). \( V_{\text{max}} \) values (from Eadie-Hofstee plots for MDPH-sensitive fluxes, in 10⁻⁶ pmol of H⁺ s⁻¹ μm⁻²) were 205 (oleic acid), 158 (myristic acid), 169 (heptylbenzoic acid), and 62 (12-azidolauroic acid), while \( K_m \) values were 72 μM for oleic, 89 μM for myristic, 207 μM for heptylbenzoic, and 384 μM for 12-azidolauroic acid.

Fig. 7. Photoaffinity labeling of rat heart mitochondria with ³H-AzHA. Photographs of SDS-PAGE (left panel) and the corresponding autoradiograms (right panel) of rat heart mitochondria (two samples, A and B), photolabeled with ³H-AzHA (3 μmol/mg protein) are shown. The positions of the molecular mass (M) standards are marked by the displayed scale. The Laemmli system of SDS-PAGE using 17.5% acrylamide with an acrylamide/bisacrylamide ratio of 150:1 was employed, and autoradiography was performed as described under “Experimental Procedures.”
Cibacron blue affinity agarose column (BS column) and still retained the $^3$H-AzHA label attached (Fig. 8a). In turn, AAC (30-kDa monomer) was tightly bound to the BS column and could be eluted as reported before (39), either at higher NaCl concentrations or with SDS (Fig. 8a). Also, the 30-kDa band in these fractions retained the $^3$H-AzHA label. It was identified as AAC by Western blots (22). The “upper” bands were found only in minute amounts in the intermediate NaCl fractions of the BS column.

When we further fractionated the BS flow-through fraction on Affi-Gel 501 (an organo-mercurial affinity matrix) and eluted first with a medium containing low and then high mercaptoethanol concentration, the intermediate fractions yielded only a 34-kDa band, which still retained the $^3$H-AzHA label (Fig. 9, sample 1). Also, when the HTP pass-through containing the labeled proteins was applied on the Affi-Gel, the resulting flow-through contained most of the typical HTP bands, among which only AAC was labeled with $^3$H-AzHA (not shown). Hence, one may expect that the protein retained in the Affi-Gel was predominantly the PIC protein (40). Identical results were obtained when rat liver or heart mitochondria was first labeled with $^3$H-AzHA and then fractionated on HTP, BS column (Fig. 8b), and Affi-Gel (Fig. 9).

Prevention of $^3$H-AzHA Photolabeling by 4-Azido-2-nitrophenylphosphate, Methanephosphonate, and Diphosphonates—To evaluate whether the P$_i$ binding domain of PIC is close or overlaps with the putative FA binding site, we performed competition studies and tested which phosphate analogs would prevent the $^3$H-AzHA photoaffinity labeling of recombinant yeast PIC. Percentages of the remaining $^3$H-AzHA label were quantified from the band density compared with controls. There was no change in the $^3$H-AzHA photolabeling when preincubations with phosphonoformate or undecanesulfonate were performed (Fig. 10). Pyrophosphate and 4-nitrophenylphosphate partly prevented the $^3$H-AzHA label. Photolabeling was prevented by preincubations with the newly identified substrate, methanephosphonate (10 mM), and was completely prevented by 10 mM methylenediphosphonate and iminodiphosphonate (methylenephosphonate) (Fig. 10). The amount of the $^3$H-AzHA label on yeast PIC also decreased after the preceding photolabeling with 4-azido-2-nitrophenylphosphate (Fig. 10).
FIG. 10. Photoaffinity labeling of recombinant yeast phosphate carrier with $^3$H-AzHA and its prevention by various phosphoanalogues. Autoradiograms of PAGE-separated yeast PIC solubilized from inclusion bodies (equal amounts, three experiments, A, B, and C) as described under "Experimental Procedures" are shown. Samples were photolabeled with $^3$H-AzHA (5.2 nmol/mg total protein, corresponding to a stoichiometry of 0.3 per dimer) in controls (C, 100%) or after preincubations with 10 mM methanephosphonate (C, Ph, remaining 37% of the label), 10 mM phosphonoformic acid (FPA, 99%), 50 mM sodium pyrophosphate (PP, 44%), 1 mM sodium undecanesulfonate (C$_{11}$SO$_3$, 99%), 10 mM methylenediphosphonate (MDPh, 9%), 10 mM iminodi(methylenephosphonate) (IDPh, 12%), 10 mM 4-nitrophenylphosphate (NPP, 40%), or after preceding photolabeling with nonradioactive 10 mM 4-azido-2-nitrophosphonate (AzNPP, 30%). For details of SDS-PAGE (17.5% acrylamide, its ratio to bisacrylamide 150:1) and autoradiography, see "Experimental Procedures."

DISCUSSION

We have demonstrated the ability of fatty acids to inhibit (strongly for azidonitrophenyl-FAs) the mitochondrial phosphate carrier (PIC) and to function as its monovalent anionic substrates in a FA cycling process. Furthermore, photolabeling of PIC with azidonitrophenyl-FAs has also been performed. In this way, we have tried to characterize the corresponding FA binding site and its relationship to the $P_i$ binding site. We have found that these two sites reside close to each other (or might overlap), since the inhibition of $P_i/P_i$ exchange by FAs is most likely of a competitive type, and covalently bound AzNPP, the newly identified substrate methanephosphonate, and the inhibitory diphosphonates did prevent $^3$H-AzHA photolabeling.

A question now arises concerning the nature of FA interaction with PIC. An identity of the FA binding site and the $P_i$ binding site could be excluded by the lack of PIC interaction with long-chain alklyphosphonates. On the contrary, a proximity of these two sites may be the origin for the observed competitive inhibition of $P_i/P_i$ exchange by FAs, for prevention of $^3$H-AzHA binding by some $P_i$ derivatives, and for the correlation between $K_i$ values of FAs inhibiting $P_i$ uptake and the apparent affinity of cycling FAs. We have intentionally selected FAs covering a range of affinities or $K_i$ values. FA cycling occurred at higher FA concentrations as suggested by higher $K_m$ values ($\sim 100 \mu M$) when compared with UCPI ($\sim 10 \mu M$; Ref. 23). The presence of a 4-azido(2-nitrophenyl) group enhanced the inhibitory ability of FAs, but it is not exclusively this portion of AzDA that mediates the inhibitory effect, since the closest analog, $\omega$-azido-dodecanoic acid, and natural FAs inhibit as well. The enhancement of the inhibitory strength could originate from the interaction within the $P_i$ binding site (anion pathway) of PIC, since AzNPP, attached by photoreaction to the carrier, does interfere with $^3$H-AzHA photolabeling. This fact again confirms the proximity of the $P_i$ and FA binding sites.

Our findings extend the previous report of Wojtczak and Zaluska (31), who interpreted the observed FA inhibition of $P_i$ transport in rat liver mitochondria as a surface charge effect. Our data indicate a rather specific effect of FAs on $P_i$ transport via PIC. A surface charge should also probably inhibit the H$^+$ efflux coupled to the Na$^+$ uptake by the Na$^+/H^+$ antiporter or the pyruvate$^*/H^+$ symport via the pyruvate carrier. None of these effects was observed. Also, several inactive FA derivatives (42, 43), such as 12-hydroxydocosenoic acid, if causing the surface charge effect, should inhibit the PIC as well. Again this was not observed. Finally, butyl- and decylphosphonate should cause even higher inhibition due to a surface charge, but they did not, even at millimolar concentrations.

Concerning the specificity of FA interaction, it is interesting to ask whether the demonstrated photolabeling of PIC with $^3$H-AzHA or FA cycling resulted from the existence of a specific, preformed, FA binding site or whether it instead reflects the overall carrier hydrophobicity and positive charges inside the membrane. This calls for further studies. Nevertheless, prior to being combusted, natural FAs will be present in the membrane due to their high partition coefficient and may potentially inhibit PIC and may cycle via PIC in either of the two cases described above. Consequently, FAs should be considered as important regulators of oxidative phosphorylation (29), since they affect PIC (Ref. 20 and this work) and AAC (18, 19, 21, 22), besides the other mitochondrial carriers (30) and uncoupling proteins (19, 23–27).

We have also revealed for the first time that the "hydrophobic" $P_i$ analog, methanephosphonate, is a good PIC substrate. This is unrelated to the FA inhibition, since alklyphosphonates with a longer chain were found not to be transported. They did not inhibit $P_i$ uptake as well. These findings suggest that the putative hydrophobic part of $P_i$ binding site cannot accommodate larger alklyphosphonate analogs. However, we found that the $P_i$ binding site also interacts with $P_i$ analogs of medium size such as methylenediphosphonate, iminodi(methylenephosphonate), and AzNPP, which inhibited $P_i$ uptake. This confirms the previous finding of inhibition by photoactivated AzNPP (32). We found that AzNPP, together with diphosphonates belongs to nontransportable $P_i$ analogs. Thus, a specific gate of PIC does not allow sulfate and tungstate to pass (7), and, on the other hand, it is able to accommodate the methyl group and part of the phosphonomethyl and phosphonomethylmino groups, or a phenyl attached to the phosphate. Wohlrab et al. (7) hypothesized that some acid residues of yeast PIC, such as Glu$^{163}$, Glu$^{164}$, Glu$^{192}$, and Glu$^{196}$ might ensure specificity for phosphonate and exclude interaction with sulfate. Interestingly, glutamate Glu$^{196}$ on UCP1 conveys the pH dependence of nucleotide di- and triphosphates binding to this protein (44). Phosphonoformate, previously reported to inhibit $P_i/P_i$ exchange (8), seems not to fit in the revealed pattern, since it is not transported and does not inhibit the net electroneutral $P_i$ transport. Perhaps the short formyl (carboxyl) group attached on phosphonate cannot cause the inhibition as methylene phosphonate does.

Diphosphonates that inhibit FA cycling enabled by PIC were also identified as the first known nontransportable substrate analogs that inhibit the putative FA anion uniport. No such inhibition was found in the case of uncoupling proteins. UCPI or PUMP-mediated FA cycling is inhibited by undecanesulfonate, which is the translocated anionic substrate (19, 23–27, 33). The existence of FA cycling via PIC indicates that this is a more general phenomenon not related exclusively to UCPs. However, it is a ligand-gated (purine nucleotide-gated) regulation of FA cycling, which is distinct for UCPs (23, 27). It is known that nucleotides, as intermediate size ligands, interact with several membrane $\alpha$-helices of UCP1 (45). Also, phosphate might interact with several trans-membrane $\alpha$-helices of PIC, as shown by the site-directed mutagenesis (7), revealing five critical res-
ides for yeast PIC spread along the whole sequence: His\textsuperscript{32} and Asp\textsuperscript{39} on the first; Glu\textsuperscript{126} and Glu\textsuperscript{137} on the second; and the Asp\textsuperscript{236} on the third trans-membrane \(\alpha\)-helix, respectively. Among them, His\textsuperscript{32}, Glu\textsuperscript{126}, and Glu\textsuperscript{137} were proposed to form a putative proton cotransport pathway (7). However, due to functional reasons, we cannot conclude that the FA binding site is identical with the \(\text{H}^+ / \text{OH}^-\) binding site of PIC, as we did for UCP1, for which FAs were documented to enter into its anion binding site (24, 25).

In conclusion, FAs interact with PIC in a hydrophobic binding site that lies in proximity to or overlaps the \(\text{Pi}\) binding site, which might represent a slightly hydrophobic internal domain in PIC. When amphiphiles such as AzDA or native FAs interfere with the domain, the transport process is inhibited. Upon interaction with PIC, FAs might also reach the opposite side of the membrane, which leads to FA cycling and uncoupling. Both inhibitory and cycling effects could lead \textit{in vivo} to a fine regulation of oxidative phosphorylation efficiency.

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