

Photoactivated Azido Fatty Acid Irreversibly Inhibits Anion and Proton Transport through the Mitochondrial Uncoupling Protein*

(Received for publication, October 21, 1995, and in revised form, November 30, 1995)

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The protonophoretic function of uncoupling protein (UCP) is activated by fatty acids. According to the “docking site” hypothesis (Ježek, P., and Garlid, K. D., *J. Biol. Chem.* 265, 19303–19311, 1990), the fatty acid binding site is identical with the anion channel of UCP. Skulachev (Skulachev, V. P. (1991) *FEBS Lett.* 294, 158–162) extended this hypothesis by suggesting that fatty acid anions are transported by UCP and that H⁺ are delivered by back-diffusion of the protonated fatty acid through the lipid bilayer. In this model, UCP does not transport H⁺ at all but rather enables fatty acids to act as cycling protonophores. New evidence supports this mechanism (Garlid, K. D., Orosz, D. E., Modrianský, M., Vassanelli, S., and Ježek, P. (1996) *J. Biol. Chem.* 271, 2615–2620).

To help elucidate these hypotheses, we synthesized a photoreactive analog of dodecanoic acid, 12-(4-azido-2-nitrophenylamino)dodecanoic acid (AzDA), and studied its effect on transport in mitochondria and proteoliposomes. AzDA behaved in every respect like a typical fatty acid. In micromolar doses, AzDA activated H⁺ translocation and inhibited Cl⁻ and hexanesulfonate uniport through UCP. After UV light exposure, however, activation of H⁺ transport was inhibited, whereas inhibition of anion transport was preserved. These effects were irreversible. Photolabeling of mitochondria with [³H]AzDA resulted in a prominent 32 kDa band of UCP, and few other proteins were labeled. The results indicate that AzDA can be ligated to the protein at or near the docking site, causing irreversible inhibition of both H⁺ and anion transport. The finding that fatty acid-induced H⁺ transport disappears along with anion transport supports the fatty acid-protonophore mechanism of H⁺ transport by UCP.

nonshivering thermogenesis and energy dissipation (1). The terminal heat-producing unit is the mitochondrial uncoupling protein (UCP), which allows for regulated short-circuiting of the protonmotive force. UCP-mediated uncoupling is inhibited by purine nucleotides and activated by fatty acids (1). Nicholls and co-workers (2–6) showed that FA activation of H⁺ conductance requires long chain (C > 10), unesterified FA with an optimum response at 14 carbons (myristic acid) and increasing response with degree of unsaturation. Activation of GDP-sensitive H⁺ transport by palmitate (7, 8) and laurate (9) has also been demonstrated with reconstituted UCP, which manifests a similar pattern for FA structure and chain length (10).

UCP also transports anions, and a newly discovered class of monovalent anionic transport substrates of UCP includes amphiphiles such as alkylsulfonates, which are translocated much faster than smaller, hydrophilic anions such as Cl⁻ (11, 12). Alkylsulfonates also competitively inhibit Cl⁻ flux through UCP (11). Because alkylsulfonates are analogues of FA, we hypothesized that the anion pathway in UCP plays the role of a FA docking site, the site for activation of H⁺ flux (11, 13). From this site, FA either act as local buffers to facilitate H⁺ transport through UCP (10), or they are translocated directly to the other side to complete half of a cycling protonophoretic cycle (9, 14, 15). Although FA must interact directly with UCP to activate H⁺ transport, no FA binding site has been detected (2). Recently, Ježek and Freisleben (16) and Ježek *et al.* (17, 18) overcame this deficiency by using EPR to detect binding of spin-labeled FA to UCP.

To further explore the interaction of FA with UCP, we synthesized a photoreactive analogue of dodecanoic acid, 12-(4-azido-2-nitrophenylamino)dodecanoic acid (AzDA) and studied its effect on H⁺ and anion transport in BAT mitochondria as well as in proteoliposomes containing reconstituted UCP. In micromolar doses, AzDA activated H⁺ translocation as intensely as palmitic acid and also inhibited anion transport through UCP. After photoreaction by UV light, activation of H⁺ transport was prevented, while anion transport was more strongly inhibited. Furthermore, both of these effects were irreversible. UCP was also photolabeled by [³H]AzDA with high specificity relative to other mitochondrial proteins.² Our interpretation of these results is that azido-FA can be immobilized in its docking site in UCP (in the anion channel) and that this site must be responsible for anion transport and H⁺ translocation. These results are also compatible with the suggested FA protonophore mechanism for UCP-mediated H⁺ transport (9, 14, 15).

Brown adipose tissue (BAT)¹ is an organ specialized for

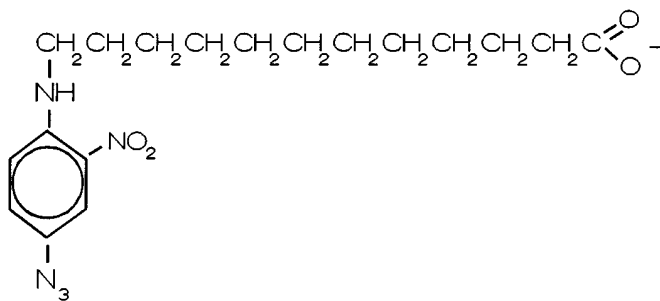
* This work was supported by National Institutes of Health (NIH) Grant GM31086 (to K. D. G.), Fogarty International Research Collaboration Award TW00120 through the NIH, and Grant 301/95/0620 from the Grant Agency of the Czech Republic (to P. J.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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¹ The abbreviations used are: BAT, brown adipose tissue; UCP, uncoupling protein; FA, fatty acid(s); TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; AzDA, 12-(4-azido-2-nitrophenylamino)dodecanoic acid; BSA, bovine serum albumin, essentially fatty acid-free; SPQ, 6-methoxy-*N*-(3-sulfopropyl)quinolinium; PBFI, potassium-binding benzofuran isophthalate; TEA, tetraethylammonium; MES, 2-(*N*-mor-

pholino)ethanesulfonic acid.

² A preliminary report of the radiolabeling experiments has been presented in abstract form (26).



SCHEME 1.

EXPERIMENTAL PROCEDURES

Synthesis of 12-(4-Azido-2-nitrophenylamino)dodecanoic Acid

Synthesis of 12-(4-azido-2-nitrophenylamino)dodecanoic acid (Scheme 1) was adapted from Bisson and Montecucco (19) but with improved methodology leading to a purer product. 4-Fluoro-3-nitrophenyl azide was prepared according to Fleet *et al.* (20) from 4-fluoro-3-nitroaniline (Janssen Chimica, Belgium). It was then coupled with 12-aminododecanoic acid (Janssen Chimica, Belgium) to form the resultant azido-FA. This was further purified by column chromatography on a silica gel (Merck, 0.04–0.063 mm, CHCl₃/MeOH, 9:1). Collected fractions of AzDA were then crystallized from CHCl₃-heptane. The red crystals were again dissolved in CHCl₃, and the insoluble red powder was filtered off. After evaporation to dryness, light red crystals with a melting point of 93–95 °C were obtained.

Synthesis of [³H]AzDA

12-Trifluoroacetamido-2-bromododecanoic acid was prepared first by bromination of 12-trifluoroacetamidododecanoic acid (obtained by reaction of 12-aminododecanoic acid and trifluoroacetic acid anhydride) in CCl₄ with thionylchloride and *N*-bromosuccinimide (21). The crude 2-bromoacetic acid was purified by column chromatography over silica gel (CHCl₃, 98% HCOOH, 100:1). The product was found to be pure by thin-layer chromatography (silica gel Merck 60F, CHCl₃/MeOH/NH₄OH (concentration) 8:2:0.1). To prepare tritiated AzDA, 25 μmol of the bromoacetic acid was then stirred for 1 h in 0.1 ml of MeOH containing 30 μl of concentrated ammonium hydroxide, 10 mg of palladium/BaSO₄ (10%), and tritium gas (~70% of carrier free activity). The reaction mixture was further lyophilized to dryness and dissolved in 0.2 ml of ethanol and 20% ammonium hydroxide, and the solution was heated to 60 °C for 3 h in order to hydrolyze the trifluoroacetyl group. The solvent was later evaporated *in vacuo*, and the crude 12-amino[³H]dodecanoic acid was dissolved in 0.6 ml of 50% ethanol. Further steps were similar as for AzDA. 7 mg (38 μmol) of 4-fluoro-3-nitrophenyl azide and 50 μl of triethylamine were added, and the mixture was heated to 55 °C overnight at dark and inert atmosphere. After evaporation of the solvent, the residue was separated by preparative thin-layer chromatography (silica gel Merck 60F) using the solvent system CHCl₃/MeOH (10:1). The principal orange-red band of the product was eluted with CHCl₃:MeOH (1:1). The yield was 4 mCi, specific activity was 1.14 Ci/mmol, and radiochemical purity was >97% (on thin-layer chromatography, silica gel Merck 60F, CHCl₃/MeOH/AcOH, 9:1:0.1).

Photochemical Modification of BAT Mitochondria

BAT mitochondria were isolated from Syrian hamsters by standard procedures modified by the addition of 2 mg/ml BSA into the isolation medium. For preincubation with FA, BSA was omitted from the final washing and stock suspension. A typical protocol for photomodification by 12-(4-azido-2-nitrophenylamino)dodecanoic acid (with preincubation and reisolated) was as follows. BAT mitochondria (1.5 mg of protein) were diluted to 0.12 mg/ml in 250 mM sucrose, 5 mM K-TEES and 1 mM K-EGTA (pH 6.7), and layered onto the bottom of a 300-ml beaker. AzDA (stock in dimethyl sulfoxide) was added to a final concentration of 20 μM, and the mitochondrial suspension was incubated for 2 min while shaking at 0 °C in the dark. The dark controls were incubated for another 2 min with AzDA. Photochemical modification was done after a 2-min preincubation in the dark using a table-top commercial tanning lamp (275 watts). UV illumination was done from a controlled distance of 20 cm, directly on the surface of the mitochondrial suspension in a shaking beaker in an ice-cold bath. The time was experimentally adjusted (see "Results") to 2 min of full lamp power. Both the dark and illuminated samples were then centrifuged at 20,000 × *g* for 10 min

(4 °C) and resuspended in 0.5 ml (3 mg/ml) of ice-cold sucrose medium. Four swelling measurements were performed from each such stock of photomodified reaction mixture. The functional properties of mitochondria were preserved after UV illumination as seen from tests without added AzDA, when UV illumination did not cause any measurable protein damage or permeability changes.

Autoradiography/Fluorography

Autoradiography/fluorography of gels containing [³H]AzDA-labeled proteins of BAT mitochondria was performed after pretreatment procedure with an enhancer ENTENSIFY (DuPont NEN), while exposing plan Kodak X-Omat AR films in steel cassettes with an intensifying screen for few weeks at -55 °C. Laemmli 12% polyacrylamide gel SDS electrophoresis system was used for separation of mitochondrial proteins employing a Bio-Rad Protean II xi electrophoresis apparatus.

Swelling Measurements

Osmotic swelling of BAT mitochondria was assayed by the 360° light-scattering technique at 520 nm using a Brinkmann PC 700 probe colorimeter (22). H⁺ transport was assayed as swelling in potassium acetate medium (6). Acetate crosses membrane in a protonated form. Swelling was induced by 0.33 μM valinomycin in 40% isotonic medium containing potassium salts of 55.8 mM acetate, 5 mM TES, 0.1 mM EGTA (total [K⁺] 57.8 mM (pH 7.2)), and rotenone (0.2 μg/ml). Transport of Cl⁻ or hexanesulfonate was assayed in 40% isotonic media containing their potassium salts (55 mM), 5 mM K-TEES, 0.1 mM EGTA (pH 7.1; plus rotenone), and was induced by valinomycin. Mitochondria were resuspended to 0.1 mg/ml in these media. Potassium hexanesulfonate (containing less >1% sodium, as determined by atomic absorption) was prepared from sodium hexanesulfonate (Sigma) via ion-exchange column (Dowex, H⁺-form) yielding the acid, and subsequently neutralized with KOH. The potassium salt was then recrystallized from ethanol. The swelling rates in β-min⁻¹ were normalized for the protein content as described elsewhere (22).

Assays with the Reconstituted Uncoupling Protein

Reconstitution was performed using an established method (9, 12, 15, 23). AzDA (3.2%) was dissolved in lipids prior to vesicle formation (8) because UV illumination disintegrated vesicles, and we intended to compare the dark and UV treatment, so UV irradiation had to be performed in detergent-lipid-UcP micelles. AzDA appeared to be principally dissolved in the lipids and was retained after treatment with Bio-Beads as judged from vesicle color.

Fluorescence changes representing Cl⁻ or K⁺ transport in SPQ- or PBFI-loaded proteoliposomes, respectively, were measured and calibrated on an SLM 8000 fluorometer (SLM, Urbana, IL) as described previously (9, 12, 15, 23, 24). Protein content of proteoliposomes was estimated by the Amido Black method (25). The volume of vesicles was determined from the distribution of [¹⁴C]-TEA-Br (12). Materials for reconstitution were from sources described previously (9, 12, 15, 23, 24).

H⁺ Efflux Measurement—H⁺ efflux from proteoliposomes was followed as the counterflux (uptake) of K⁺ detected by a fluorescence indicator, PBFI (Molecular Probes, Inc., Eugene, OR) as described previously (15, 24). The adjusted dose of valinomycin (0.66 μM) ensured that only the H⁺ flux was rate-limiting in a medium devoid of permeant anions. The vesicle interior contained 59.9 mM TEA₂-SO₄, 61.8 mM MES (TEA-salt; pH 6.5), 0.6 mM EDTA (TEA-salt), 0.2 mM K₂SO₄, and 50 μM PBFI. 150 μl of vesicles (40 mg lipid/ml) were mixed with 1.85 ml of 75 mM K₂SO₄ including 0.6 mM EDTA (TEA-salt) and 24% isotonic TEA-TEES (pH 7.5). Transport was initiated by valinomycin.

Cl⁻ Uptake Measurement—Cl⁻ uptake was detected using quenching of fluorescent probe SPQ (9, 12, 15). Proteoliposomes contained 192.3 mM TES (TEA-salt, [TEA] = 61.4 mM; pH 7.2), 85.9 mM TEA₂-SO₄, 0.5 mM TEA-EGTA, and 0.14 mM KCl. External medium for the assay consisted of 129 mM KCl, 192.3 mM TES (TEA-salt, [TEA] = 61.4 mM (pH 7.2)), and 0.5 mM TEA-EGTA. 25 μl of vesicles (40.5 mg lipid/ml) containing UCP were used for one measurement. Cl⁻ uptake was initiated by valinomycin. Other conditions were as described previously (12).

RESULTS

Activation of H⁺ Transport in Brown Adipose Tissue Mitochondria by Palmitic Acid and AzDA—AzDA, with its alkyl tail encumbered by an azido group, is a modified FA, and its interaction with UCP was unknown. We carried out the H⁺ activation studies summarized in Fig. 1 in order to determine the extent to which AzDA mimics normal FA in brown adipose

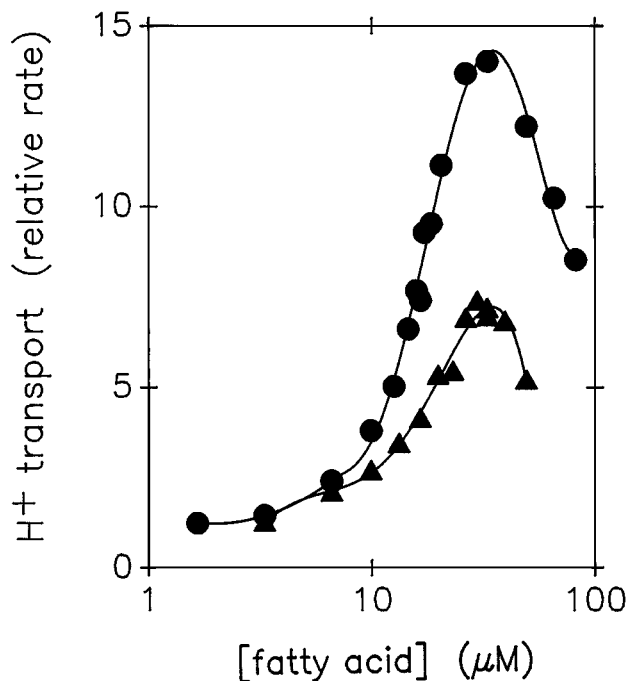


FIG. 1. Activation of UCP-mediated H^+ transport in brown adipose tissue mitochondria by palmitic acid and AzDA in the dark. The figure contains dose-responses for activation of H^+ transport by palmitic acid (●) and AzDA (▲). Half-maximum activation constants, $K_{0.5}$, were $15.9 \mu\text{M}$ (155 nmol/mg of protein) for palmitic acid and $16.6 \mu\text{M}$ (167 nmol/mg of protein) for AzDA. The maxima were taken as 100% for estimation of the K_a values. Mitochondria in 2 mg/ml BSA were diluted into KOAc assay medium. Fatty acids were added at 30 s , and $0.33 \mu\text{M}$ valinomycin was added at 1 min to activate H^+ transport and net uptake of KOAc, with osmotic swelling. H^+ transport is plotted as the ratio of the observed swelling rates to the rate in BSA (0.125 min^{-1}). Maximum rates of 1.75 and 0.93 min^{-1} were achieved with $33 \mu\text{M}$ palmitic acid and $30 \mu\text{M}$ AzDA, respectively. Rates in both fatty acids were strongly inhibited (to 0.09 min^{-1}) by 1 mM GDP.

tissue mitochondria. Addition of FA to mitochondria invariably causes dose-dependent changes in the light scattering response, but BSA-buffering minimizes this effect (9). In the experiments reported in Fig. 1, mitochondria were isolated and stored in medium containing 2 mg/ml BSA to remove endogenous FA and then diluted 1000-fold into the assay medium. Under these conditions, palmitic acid caused a 14-fold stimulation of H^+ transport in brown adipose tissue mitochondria (Fig. 1, upper curve), and AzDA caused an 8-fold stimulation of H^+ transport (Fig. 1, lower curve). Addition of more BSA to the assay medium prevented stimulation by both FA and reduced the rates to 7–10% of maximum stimulated activity. Addition of 1 mM GDP to the assay medium inhibited the activated flux by more than 95%, indicating exclusive participation of UCP in the process. (The K_i for GDP inhibition under these conditions is about $15 \mu\text{M}$ (9).) We also measured nigericin-induced swelling in KOAc medium containing 1 mM GDP. This flux is independent of protein-mediated processes and serves as an indicator of mitochondrial intactness and preservation of a constant light-scattering response. Nigericin-induced swelling was not affected by palmitic acid or AzDA, when these FA were added at $25 \mu\text{M}$ or less. From these studies, we conclude that AzDA behaves like a typical FA in BAT mitochondria and is competent to activate UCP-mediated H^+ transport.

It was also necessary to study the effects of AzDA in BSA-free medium, because BSA would interfere with photoactivation. BSA was omitted from the penultimate mitochondrial suspension, and this suspension was then centrifuged and resuspended without BSA. When AzDA or palmitic acid was preincubated with these mitochondria for 4 min at 0°C in the

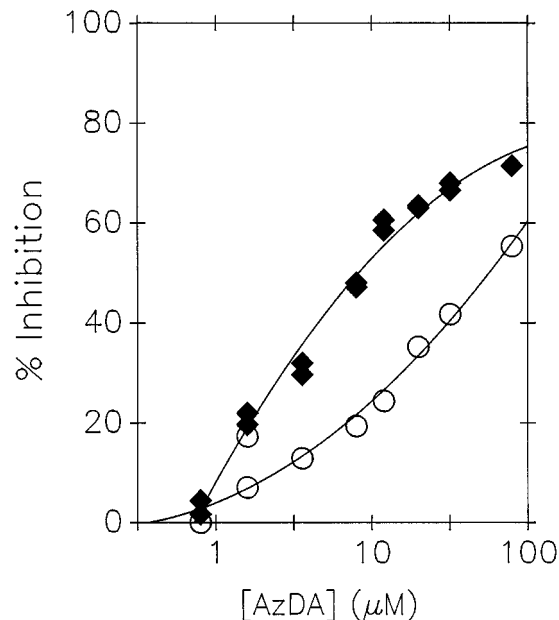


FIG. 2. Inhibition of UCP-mediated H^+ transport in brown adipose tissue mitochondria by AzDA following illumination by UV light. Shown are dose-response curves for inhibition of UCP-mediated H^+ transport (◆) and nigericin-mediated KOAc transport in the presence of 1 mM GDP (○). The latter process bypasses UCP and is taken as a gauge of the light scattering response. For both sets of data, inhibition is relative to the maximum rate observed with AzDA (1 min^{-1}) in the dark. Actually this rate was very similar to the transport rates in mitochondria isolated without BSA and containing natural levels of endogenous fatty acids. The control rate for nigericin-mediated transport was 1.5 min^{-1} . The $K_{0.5}$ for AzDA inhibition was $9 \mu\text{M}$ for H^+ transport and $70 \mu\text{M}$ for nigericin-mediated transport. Brown adipose tissue mitochondria, without BSA buffering, were first preincubated for 2 min with $20 \mu\text{M}$ AzDA in sucrose medium and then illuminated by a UV lamp for 2 min , as described under "Experimental Procedures." The assay was conducted after centrifugation and resuspension of the mitochondrial pellet in sucrose medium.

dark, H^+ transport was stimulated 8.5- and 13-fold, respectively, relative to the BSA control (data not shown). In three separate experiments, peak rates averaged 0.92 min^{-1} with AzDA. Rates without AzDA were 0.1 min^{-1} in the presence or absence of 2 mg/ml BSA. The lack of effect of BSA demonstrates the effectiveness of BSA pretreatment in removing endogenous FA. At doses less than $20 \mu\text{M}$, AzDA had no effect on nigericin-mediated swelling in KOAc medium containing 1 mM GDP.

Irreversible Inhibition of UCP-mediated H^+ Transport following Photoreaction in the Presence of AzDA—The data in Fig. 2 were obtained after photoillumination of brown adipose tissue mitochondria in the presence of AzDA. Photoreaction caused inhibition of UCP-mediated H^+ transport (Fig. 2, upper curve) but also caused apparent inhibition of nigericin-mediated transport of KOAc (Fig. 2, lower curve). Nigericin-mediated transport of KOAc bypasses UCP, and changes in these rates are taken as a gauge of nonspecific changes in the light-scattering properties of the mitochondria. Photoillumination in the absence of AzDA had little effect on FA-induced H^+ transport or nigericin-induced KOAc transport. Furthermore, the effects of photoreaction on both processes were largely prevented by preincubation with palmitic acid prior to addition of AzDA and illumination. Such competition is not expected for a nonspecific effect.

Photoreaction with AzDA caused irreversible inhibition of UCP-mediated H^+ transport. At $20 \mu\text{M}$ AzDA, residual H^+ transport was 0.35 min^{-1} (no BSA) and 0.18 min^{-1} (with BSA). The difference amounts to 20–25% of that observed without photoreaction. Thus, activation of basal rates dropped from

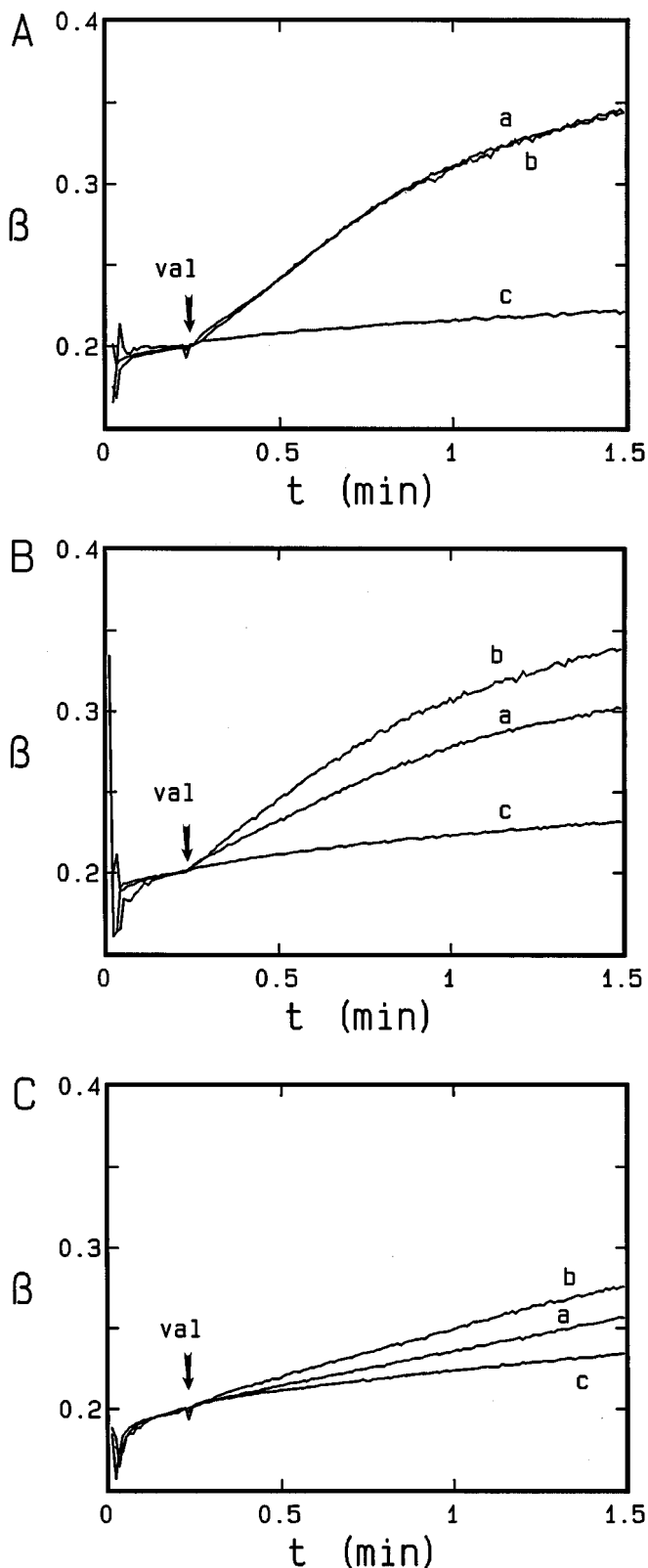


FIG. 3. Inhibition of UCP-mediated Cl^- uniport in BAT mitochondria by AzDA. An upward deflection of the light scattering parameter, β , is caused by matrix swelling secondary to KCl influx. *Panel A*, no AzDA; *panel B*, $20 \mu\text{M}$ AzDA; *panel C*, $20 \mu\text{M}$ AzDA plus 2 min of UV illumination. In all panels, *traces a* were measured with no additions; *traces b* were measured with 2 mg of BSA/ml; and *traces c* were measured with 1 mM GDP. $0.33 \mu\text{M}$ valinomycin (*val*) was added as indicated. Brown adipose tissue mitochondria, without BSA buffering, were assayed before (*panel A*) or after (*panel B*) preincubation for 2 min with $20 \mu\text{M}$ AzDA in sucrose medium or after (*panel C*) illumination in the presence of $20 \mu\text{M}$ AzDA by a UV lamp for 2 min. The assay was

8.5–13-fold activation to 1.6–2.2-fold activation. It was reassuring to observe that the residual H^+ transport, due to unreacted AzDA, was still inhibited by BSA and GDP.

Inhibition of H^+ transport decayed exponentially with time of illumination, yielding a time constant of about 55 s in $20 \mu\text{M}$ AzDA. 120 s was chosen for standard illumination duration because a sharp decrease in nigericin-induced transport occurred at longer times.

Reversible and Irreversible Inhibition of UCP-mediated Cl^- Transport by Azido Fatty Acid—Given that AzDA supports UCP-mediated H^+ transport (Fig. 1), the docking site hypothesis (11, 13) predicts that AzDA should also inhibit UCP-mediated anion transport. We confirmed this prediction in experiments using BSA-buffered mitochondria as described in the legend to Fig. 1 (data not shown). UCP-mediated Cl^- uniport was assayed by light scattering in KCl medium. When 20 – $30 \mu\text{M}$ AzDA was added to the assay medium, Cl^- uniport was completely inhibited to the level observed with 1 mM GDP. This inhibition was prevented when the assay medium was supplemented with 2 mg/ml BSA.

BSA buffering is not suitable for photoreaction, and Fig. 3 contains a study of KCl uptake in the absence of BSA buffering. In the absence of AzDA, Cl^- exhibits the normal transport profile exemplified by the three traces in Fig. 3A. Electrophoretic Cl^- uptake was initiated by valinomycin (*trace a*); BSA had little effect (*trace b*), indicating thorough removal of endogenous FA; and 1 mM GDP inhibited nearly completely (*trace c*). The three traces in Fig. 3B were obtained in the same way, except that mitochondria were first preincubated with $20 \mu\text{M}$ AzDA in the dark. Cl^- uniport was inhibited by only 34% (*trace a*), and this inhibition was reversed by addition of BSA to the assay medium (*trace b*). Partial inhibition by AzDA is due to the fact that mitochondria were reisolated after exposure to AzDA. 1 mM GDP inhibited nearly completely (*trace c*).

The three traces in Fig. 3C show that UV illumination in the presence of $20 \mu\text{M}$ AzDA caused nearly complete inhibition of Cl^- uniport. BSA reversed part of this effect, presumably due to the presence of unreacted AzDA. The data in Fig. 3C are directly comparable with those in Fig. 3B because illumination in the absence of AzDA did not affect Cl^- transport (not shown).

Fig. 4 contains the normalized dose dependence of the effects of AzDA on UCP-mediated Cl^- uniport in the presence (Fig. 4, + UV) and absence (Fig. 4, dark) of UV illumination. The effects of AzDA in the dark were obtained with AzDA added directly to the assay medium in the absence of BSA. Rates were normalized to protein-independent swelling rates in order to correct for dose-dependent, nonspecific effects of AzDA, which were higher in the absence of BSA, but independent of illumination. Protein-independent swelling rates were measured in KCl medium with nigericin, tributyltin, and GDP. 0% inhibition was set equal to normalized rates of samples preincubated and assayed with BSA and without AzDA, and 100% inhibition was set equal to the rates in 1 mM GDP, whose K_i is about $15 \mu\text{M}$. The same controls were used for both curves.

In the dark, AzDA inhibition of Cl^- transport exhibited an apparent K_i of $6.9 \mu\text{M}$ and a Hill coefficient of 1.6. Without correction for the nonspecific effect, the K_i was $5.9 \mu\text{M}$, and the Hill coefficient was 2.2. AzDA inhibition in the dark was fully reversible by BSA. A dose-response curve in the presence of constant BSA (2 mg/ml) yielded an apparent K_i of over $100 \mu\text{M}$. After illumination, AzDA inhibition of Cl^- transport exhibited

conducted after centrifugation, and resuspension of the mitochondrial pellet in sucrose medium. Assay medium contained 55 mM KCl, and uptake of KCl with swelling required both valinomycin and UCP.

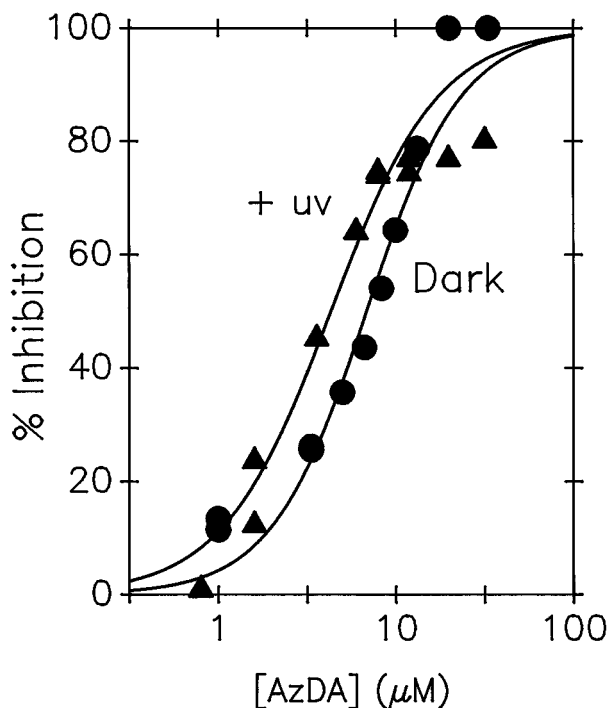


FIG. 4. Inhibition by AzDA of UCP-mediated Cl^- uniport with (\blacktriangle) and without (\bullet) UV illumination. Percent inhibition of GDP-sensitive KCl uptake into brown adipose tissue mitochondria is plotted versus [AzDA]. Data obtained without illumination (*Dark*) used BSA-buffered mitochondria (preincubation of mitochondrial stock with 2 mg/ml BSA, followed by 1000-fold dilution into assay medium), and AzDA was added to the assay medium in the indicated doses. Data obtained with illumination (+ *UV*) used unbuffered mitochondria preincubated with indicated doses of AzDA, illuminated for 2 min, and reisolated by centrifugation prior to the light scattering assay. Valinomycin was added during assay to initiate KCl uptake in 55 mM KCl medium. The swelling rate is limited under these conditions by UCP-mediated Cl^- influx. Measured swelling rates were first normalized to protein-independent swelling rates, as measured in the same KCl medium containing 0.33 μM nigericin, 3.3 μM tributyltin chloride, and 1 mM GDP. This is intended to correct for nonspecific, dose-dependent effects of AzDA on the two mitochondrial preparations. For both sets of normalized data, 0% inhibition was set equal to values obtained in samples preincubated and assayed with BSA, and 100% inhibition was set equal to values obtained in 1 mM GDP, whose K_i is about 15 μM . As described in the text, AzDA inhibition after photoreaction was not reversed when 2 mg/ml BSA was added to assay medium, whereas BSA did prevent inhibition in the absence of photoreaction.

a K_i of 4.3 μM and Hill coefficient of 1.4 (Fig. 4). Without correction for the nonspecific effect, the K_i was 2.4 μM and the Hill coefficient was 1. This effect was *not* reversible by 2 mg/ml BSA added to the assay medium.

Reversible and Irreversible Inhibition of UCP-mediated Hexanesulfonate Transport by Azido Fatty Acid—The effects of AzDA on hexanesulfonate transport were similar to those on Cl^- transport. In BSA-buffered mitochondria, 20–30 μM AzDA inhibited hexanesulfonate uniport to the level observed with 1 mM GDP, and this inhibition was prevented by 2 mg/ml BSA in the assay medium. Following preincubation with 20 μM AzDA in the dark and reisolation of the mitochondria, potassium hexanesulfonate uptake was inhibited by only 19%, whereas nearly complete inhibition occurred after UV illumination (data not shown).

Fig. 5 contains dose-response curves for inhibition of UCP-mediated hexanesulfonate uniport by AzDA added directly to the assay medium (*left panel*) and after preincubation, UV illumination, and reisolation (*right panel*). AzDA inhibition of hexanesulfonate transport in the dark exhibited a K_i of 4.8 μM and a Hill coefficient of 1.7. Reversibility of inhibition in the

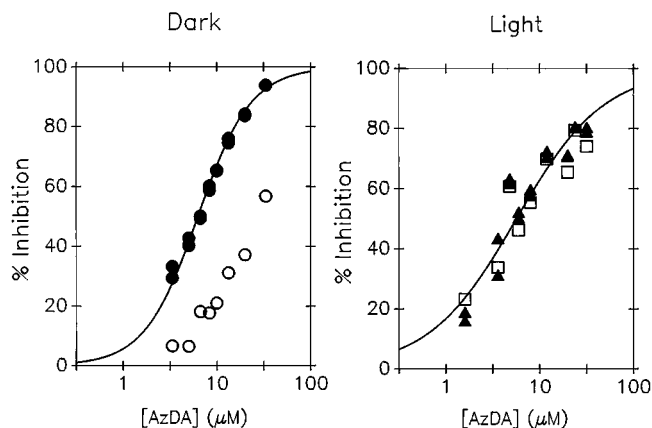


FIG. 5. Inhibition by AzDA of UCP-mediated hexanesulfonate uniport without (\circ) UV illumination. Percent inhibition of GDP-sensitive potassium hexanesulfonate uptake into brown adipose tissue mitochondria is plotted versus [AzDA]. *Left panel*, without illumination: \bullet , mitochondria in 2 mg/ml BSA were diluted 1000-fold into potassium hexanesulfonate assay medium, and AzDA was added in the indicated doses; \circ , parallel experiments with 2 mg/ml BSA added to medium. *Right panel*, after illumination: \blacktriangle , mitochondria were preincubated with indicated doses of AzDA in the absence of BSA, illuminated for 2 min, and reisolated by centrifugation prior to the light scattering assay; \square , parallel experiments with 2 mg/ml BSA added to medium. Assay conditions and calculation of AzDA inhibition were identical to procedures described in the legend to Fig. 4, except that assay medium contained 55 mM potassium hexanesulfonate instead of KCl.

dark is demonstrated by the dose-response curve measured with 2 mg of BSA/ml, which shifted the apparent K_i to over 40 μM (Fig. 5, *open circles*). After photoactivation (Fig. 5, *right panel*), AzDA inhibited hexanesulfonate uniport with a K_i of 5.7 μM and Hill coefficient of 1. Irreversibility of this inhibition is demonstrated by the lack of effect of 2 mg/ml BSA on inhibition (Fig. 5, *open squares*).

Effects of Azido Fatty Acid in Proteoliposomes Reconstituted with UCP—We studied the effects of AzDA on three preparations: normally prepared proteoliposomes, proteoliposomes supplemented with 3.2% AzDA (w/w lipid) during reconstitution, and proteoliposomes supplemented with 3.2% AzDA and exposed to UV illumination for 2 min before their formation. UCP was isolated from BAT mitochondria in the presence of BSA to remove endogenous FA. When added directly to the assay cuvette containing normally prepared proteoliposomes, AzDA activated H^+ transport ($K_a \approx 15 \mu\text{M}$) and inhibited Cl^- transport ($K_i \approx 39 \mu\text{M}$). Addition of AzDA was also associated with electroneutral proton delivery to the internal medium, showing that protonated AzDA is capable of nonionic flip-flop across the bilayer. These data are not shown, because AzDA behaved in every respect like laurate or other FA when similar protocols were used (8, 9, 15).

Fig. 6 contains data obtained from proteoliposomes supplemented with AzDA *before* UV illumination. This protocol was used as a control for the illumination experiments. As illustrated by the data in Fig. 6A, H^+ transport was stimulated when compared with control proteoliposomes; however, a large component of AzDA-activated H^+ transport is attributable to increased proton leak in these specially prepared proteoliposomes. 1 mM GDP inhibited about 50% of the BSA-sensitive flux (Fig. 6A, +AzDA +GDP). 50% inhibition by external GDP is the normal value for FA-activated H^+ transport, because GDP inhibits from one side of UCP, and our reconstitutions contain randomly oriented UCP (9, 12, 15). Accordingly, it was most appropriate to compare the GDP-sensitive rates of H^+ transport, calculated as the difference between rates in the presence and absence of 1 mM GDP. On average, GDP-sensitive

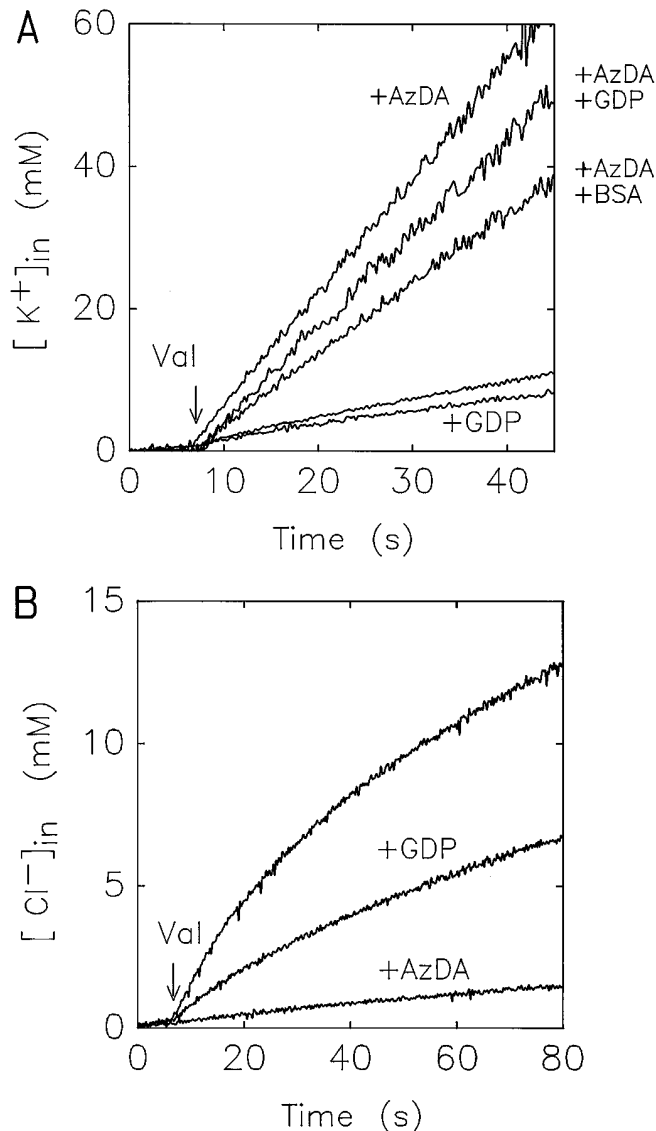


FIG. 6. Effects of AzDA in proteoliposomes reconstituted with UCP. UCP was isolated from brown adipose tissue mitochondria in the presence of BSA to minimize endogenous fatty acids. To prepare for photoreaction, lipids were supplemented with 3.2% AzDA (w/w lipid). Data shown were obtained *before* UV illumination. *Panel A*, activation of H^+ transport by AzDA. Internal $[K^+]_{in}$ in proteoliposomes reconstituted with purified UCP was measured using the fluorescence probe, PBFI. Under conditions of these experiments, K^+ influx is a measure of H^+ efflux. Electrophoretic H^+ flux was initiated by adding $0.66 \mu M$ valinomycin (*val*). The *lower two traces* were obtained from proteoliposomes without AzDA in the presence (+GDP) or absence (unlabeled traces) of 1 mM GDP. The low rate of GDP-sensitive H^+ efflux confirms that most endogenous fatty acids are removed by the purification/reconstitution protocols. The upper three traces were obtained from proteoliposomes supplemented with AzDA. +AzDA, no further additions; +AzDA+GDP, 1 mM GDP added to assay medium; +AzDA+BSA, 2 mg/ml BSA added to assay medium. *Panel B*, inhibition of Cl^- transport by AzDA. Internal $[Cl^-]_{in}$ in proteoliposomes reconstituted with purified UCP was measured using the fluorescence probe, SPQ. Electrophoretic Cl^- flux was initiated by adding $0.66 \mu M$ valinomycin (*val*). *Upper trace*, no AzDA; +GDP, no AzDA, 1 mM GDP added to assay medium; +AzDA, proteoliposomes reconstituted with 3.2% (w/w lipid) AzDA.

H^+ flux was stimulated 4.2-fold by 3.2% (w/w lipid) AzDA; differential rates were 3800 nmol of H^+ /min mg of protein with AzDA versus 900 nmol H^+ /min mg of protein without AzDA. Following UV illumination in the presence of AzDA prior to vesicle formation, GDP-sensitive H^+ flux in the vesicles was sharply reduced, to 1080 nmol of H^+ /min mg of protein. This

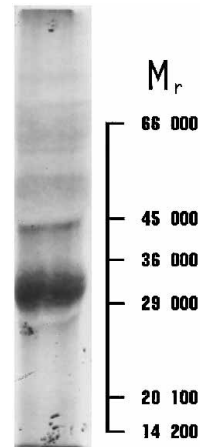


FIG. 7. Photoaffinity labeling of brown adipose tissue mitochondria with $[^3H]AzDA$. Shown is an autoradiograph of mitochondrial proteins following photolabeling with $[^3H]AzDA$. Mitochondria were illuminated for 2 min in UV light in the presence of 125 nmol/mg of protein ($143 \mu Ci$) $[^3H]AzDA$, washed in 0.25 M sucrose, and recovered by centrifugation. Proteins were separated on 12% SDS-PAGE, and the gel was treated with an enhancer for autoradiography.

flux was no longer sensitive to BSA added to the assay medium.

Fig. 6B contains data using the same protocols showing that AzDA inhibits Cl^- transport nearly completely (91–94%) in proteoliposomes containing UCP. Again, GDP inhibited Cl^- flux by about 50%. GDP is a partial inhibitor, because the nucleotide binding sites are randomly oriented during reconstitution (9, 12, 15). AzDA inhibited Cl^- transport nearly completely, because transported anions and FA compete for the same site on UCP and are mutually competitive inhibitors (9, 15). AzDA inhibition of Cl^- flux, like FA inhibition of Cl^- flux (9), was partially reversed by 2 mg/ml BSA added to the assay medium (not shown). Following UV illumination in the presence of AzDA, inhibition became irreversible (no longer affected by BSA). These findings with reconstituted UCP confirm findings in brown adipose tissue mitochondria. Without UV illumination, AzDA reversibly activated H^+ transport and inhibited Cl^- transport; with UV illumination, AzDA irreversibly inhibited both processes.

$[^3H]AzDA$ Photolabeling of Uncoupling Protein in BAT Mitochondria—Using $[^3H]AzDA$ in the photoillumination protocol, we evaluated which of the membrane proteins of brown adipose tissue mitochondria can be photolabeled with AzDA. UCP was labeled, as shown by the very prominent band corresponding to M_r 32,000 (Fig. 7). Surprisingly little labeling of other proteins was observed. After extensive washing following photomodification, about 36% of the measured radioactivity recovered from brown adipose tissue mitochondria was bound to UCP.

DISCUSSION

Our work represents a further attempt to demonstrate the existence of a specific binding site for FA on the mitochondrial uncoupling protein and to probe its properties. The azido-FA, AzDA, behaved in the dark like other FA; it catalyzed H^+ transport and inhibited transport of Cl^- and hexanesulfonate. In parallel with covalent photolabeling of UCP with AzDA, we performed functional tests to determine in which functional domains AzDA affects UCP. We observed that photomodification with AzDA inhibits both H^+ transport and anion transport. Specificity of the observed effects was confirmed by photolabeling of BAT mitochondria with $[^3H]AzDA$, which showed prominent labeling of a M_r 32,000 band corresponding to UCP. Thus, our data support the hypothesis (11) that the anion transport domain and the FA interaction domain are identical.

Our previous work indicated that the anion transport pathway in UCP is a hydrophobic channel, a suggestion deduced from the fact that the transport rate increases with hydrophobicity of alkylsulfonates (11). Hydrophobic domains in the transport pathway have also been suggested for other mitochondrial membrane porters (27). Nontransported alkylsulfonates, generally those with additional polar groups, failed to inhibit anion transport, suggesting that an external binding site was either absent or shielded from the aqueous medium (11, 15). The hydrophobic anion channel must contain an energy well to lower the barrier to transport, and we proposed that this internal energy well is identical with the FA binding site on UCP. The flux-voltage profile for Cl^- transport through UCP indicated that this energy well was located near the center of the membrane and surrounded by two hydrophobic energy barriers (13). We have recently presented evidence that this anion channel in UCP is in fact designed to conduct FA anions across the membrane (15).

A central question is whether the energy well in the anion channel is identical with the fatty acid carboxylate interaction domain, as predicted by the docking site hypothesis (11, 13). This hypothesis states that the anion transport properties of UCP are secondary consequences of the FA binding site, which was phylogenetically developed to permit H^+ translocation. Simultaneous inhibition of anion transport and activation of H^+ transport by AzDA supports overlapping of the FA docking site with the anion channel energy well. Moreover, competitive inhibition of anion transport by FA excludes the possibility that FA inhibition is allosteric (9). Further evidence favoring the docking site hypothesis is provided by the fact that the irreversibly photoattached AzDA to UCP blocks both H^+ transport and anion transport.

The docking site hypothesis is consistent with either mechanism of H^+ transport through UCP. (i) The anion channel is designed to transport FA anions across the membrane, and the concomitant return of protonated FA leads to FA cycling and electrophoretic H^+ transport (9, 13–15). (ii) The energy well is designed for FA binding, and FA carboxylates provide a local buffer to facilitate H^+ transport through a putative H^+ channel in UCP (10). AzDA is a 12-carbon FA, a length at which maximum H^+ flux is observed (10). Photomodification with AzDA should enable the carboxylate group to move freely (Scheme I), and may be expected to enhance H^+ transport. In fact, the opposite was observed. This result cannot exclude the FA buffering model but also does not support it. On the other hand, our results are fully predicted by the FA protonophore model (14, 15). Thus, photomodification with AzDA irreversibly inhibits H^+ and anion transport and also reduces the ability of unreacted AzDA to induce H^+ transport. Clearly, a FA cycling mechanism cannot proceed if a FA is covalently attached to the channel.

H^+ transport through UCP can be activated by FA, such as AzDA and others (16–18), that contain a bulky hydrophobic group. This indicates that the channel is large enough to accommodate more than a linear aliphatic chain. It seems likely that this accommodation is achieved by virtue of the fact that the channel lies at the protein-lipid interface (11, 15). In soluble proteins, hydrophobic clefts contain some charged groups to orient FA carboxyl groups (28, 29). Three out of six membrane-spanning loops of UCP contain positive charges (30). If one or more of these α -helices comprise part of the anion channel, we

would expect that they would be accessible from the lipid hydrocarbon core.

The capacity of UCP to catalyze uncoupled respiration has led to the suggestion that brown adipose tissue normally functions to prevent human obesity (31). In support of this idea, ablation of brown adipose tissue in transgenic mice resulted in obesity and increased energy efficiency (32). New evidence supports the idea that a defect in energy dissipation plays an important role in human obesity and its sequel, a non-insulin-dependent diabetes mellitus. Thus, a specific mutation of the β_3 -adrenergic receptor correlates with obesity, and it has been suggested that the role of this mutation in the pathogenesis of obesity is to lower the resting metabolic rate (33). The role of the β_3 -receptor in stimulating energy-dissipation via UCP in BAT mitochondria is well established (34). It is therefore reasonable, but still speculative, to suggest that UCP plays a role in regulation of normal body weight in human adults. This emphasizes the importance of resolving the mechanism of FA-induced H^+ transport by UCP and its regulation.

Acknowledgments—We thank David E. Orosz for help with the reconstitution experiments.

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