Photoaffinity labelling of the mitochondrial uncoupling protein by [^{3}H]azido fatty acid affects the anion channel

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Abstract Brown adipose tissue (BAT) mitochondria were incubated with the azido derivative of fatty acid (hexadecanoic) containing four tritium atoms, [^{3}H]AzHA, and among all mitochondrial proteins only a few proteins were photolabelled after irradiation with UV. It suggests the existence of specific fatty acid binding sites on mitochondrial proteins. It was also possible to label with [^{3}H]AzHA the isolated uncoupling protein (UcP) of BAT mitochondria with a low stoichiometry — lower than one AzHA per dimeric UcP. These results together with the observed competition (i.e. prevention of photolabelling) of various UcP anionic substrates with [^{3}H]AzHA and its dodecanoic acid analogue, suggest the existence of the specific fatty acid binding site on UcP identical with the anion channel or anion translocating site.

Key words: Uncoupling protein; Photoaffinity labelling; Azido fatty acid; Fatty acid

1. Introduction

The uncoupling protein (UcP) belongs to the most studied anion transporters as a member of the gene family of homologous mitochondrial proteins [1,2]. UcP function [3–8] is the uniport of monovalent unipolar anions [7] including the uniport of the anionic forms of fatty acids [3–6]. The latter leads to the fatty acid cycling uncoupling mechanism originally suggested by Skulachev for the ADP/ADP antiporter and UcP [9] (see also [3] and [10]). Within the framework of this concept, UcP conducts fatty acid anion and return of protonated fatty acid (FA) enables proton transport across the membrane.

Previous results of Ježek et al. [4] demonstrated the ability of azido derivative of dodecanoic acid to inhibit anion transport via uncoupling protein while at the same time this azido FA provided the equal stimulation of protonophoric activity of UcP as did palmitic acid. Moreover, after UV irradiation inhibition of anion transport became stronger, whereas the protonophoric activity was reduced. Also recent competition studies [3] between lauric acid and undecanesulfonate, transport of which via UcP was verified, suggested that a putative FA binding site is identical with the anion translocation pathway (a channel) of UcP. EPR spectroscopy of 5-DOXYL-stearic acid [5,6] indicated the existence of such a binding site and also showed competition of this spin-labelled FA with UcP substrates, alkylsulfonates [5].

In this work we have synthesized an azido derivative of hexadecanoic acid with very high specific radioactivity, i.e. 16-(4-azido-2-nitrophenylamino)[^{3}H$_4$]hexadecanoic acid, containing four tritium atoms ([^{3}H]AzHA), and probed for the existence of FA binding sites in BAT mitochondria and on isolated UcP. We have found that among all mitochondrial proteins only a few proteins can be labelled with [^{3}H]AzHA, which suggests the existence of specific fatty acid binding sites on mitochondrial proteins. The specific FA binding site on UcP has been labelled with low stoichiometry and competition with alkylsulfonates has been demonstrated. Preliminary data were presented as a congress report [11].

2. Materials and methods

Brown adipose tissue (BAT) mitochondria were isolated from Syrian hamsters in a medium of 250 mM sucrose, 10 mM Tris-MOPS, 1 mM Tris-EGTA, pH 7.2, containing 5 mg BSA/ml. Last washing was done in medium without BSA. Uncoupling protein was isolated as described by Ježek and Freisleben [6]. Most of the chemicals were purchased from either Sigma or Fluka. Octylpentaoxyethylene was from Bachem (Bubendorf, Switzerland), octadecanesulfonate was from Lancaster (Germany) and precursors for azido fatty acid synthesis were from Janssen Chimica (Belgium) and Aldrich (USA). ENTENSIFY, NEF-992, a universal autoradiography enhancer has been repeatedly delivered by NEN-DuPont (USA). [^{3}H]AzDA and nonradioactive AzDA were prepared as described previously [4].

2.1. Synthesis of azidohexadecanoic acid containing four tritium atoms

[^{3}H]AzHA, i.e. 16-(4-azido-2-nitrophenylamino)[^{3}H$_4$]hexadecanoic acid was synthesized as follows. First, 16-aminohexadec-8(9)-enoic acid was prepared from 8-cyclohexadecen-1-one (Aldrich, USA) similarly to the method described previously for 16-aminohexadecanoic acid [12]. Then, 16-amino[^{3}H]hexadecanoic acid was prepared by catalytic hydrogenation: 21 μmol KOH as 1 N methanolic solution was added to 20 μmol (5.4 mg) of 16-aminohexadec-8(9)-enoic acid and 10 mg 10% Pd/$BaSO_4$ in 0.5 ml of methanol. The acid was hydrogenated with tritium gas (approx. 85% of carrier-free radioactivity) at 700 Torr for 1 h. The reaction mixture was freeze-dried, then dissolved in 0.7 ml methanol and the catalyst was removed by centrifugation. The crude 16-amino[^{3}H]hexadecanoic acid (51.8 GBq) showed 80–85% radiochemical purity (TLC: silica gel Merck 60 F_{254}, $CHCl_3$-MeOH-AcOH 20 : 1 : 0.5) and was further used without purification.

16-(4-Azido-2-nitrophenylamino)[^{3}H$_4$]hexadecanoic acid preparation: 51.8 GBq (1400 mCi) of the crude 16-aminohexadecanoic acid in 0.3 ml methanol was stirred with 25 μmol (4.55 mg) of 4-fluoro-3-nitrophenyl azide and 5 ml triethylamine at 60°C for 16 h (under inert atmosphere in dark). The orange-red reaction mixture was evaporated in vacuum and separated preparatively on silica gel (Merck 60 F_{254}, 0.2 mm, 20×20 cm, $CHCl_3$-MeOH-AcOH 10:1:0,5, eluted 2×). The principal radioactive bands with R_f of 0.2 and orange-red with R_f of 0.8 were eluted with methanol giving 4.4 GBq of pure 16-aminohexadecanoic acid and 18.1 GBq of 16-(4-azido-2-nitrophenylamino)hexadecanoic acid. The azido acid was rechromatographed on silica gel

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Abbreviations: [^{3}H]AzDA, 12-(4-azido-2-nitrophenylamino)-[^{3}H]dodecanoic acid; [^{3}H]AzHA, 16-(4-azido-2-nitrophenylamino)[^{3}H$_4$]hexadecanoic acid; BAT, brown adipose tissue; BSA, bovine serum albumin; FA, fatty acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; Octyl-POE, octylpentaoxyethylene; UcP, uncoupling protein

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(Merck 60 F_{254}, 0.2 mm, 12×20 cm, $CHCl_3$-MeOH-AcOH 20:1:0.5). The orange-red band was eluted with methanol giving the total radioactivity of 9.4 GBq (255 mCi) and radiochemical purity >97% (HPTLC: silica gel Merck 60 F_{254}, $CHCl_3$-MeOH-conc. NH_4OH 8:2:0.2). Specific radioactivity of 4.8 TBq/mmol (130 Ci/mmol) was determined by spectrophotometry (260 nm and 465 nm).

2.2. Protocol for photolabelling of BAT mitochondria with [^{3}H]AzHA

3 mg of BAT mitochondria were resuspended in 10 ml of BSA-free sucrose isolation medium. [^{3}H]AzHA was added to a final concentration of 0.46 or 4.68 μM corresponding to 1.5 or 15.3 nmol/mg mitochondrial protein, respectively. This accounted for molar ratio of [^{3}H]AzHA : dimeric UcP approximately 1.43:1 or 14.3:1, respectively (when UcP amount is roughly estimated as 7% of total mitochondrial protein). The mixture was then shaken first in darkness for 4 min and then illuminated for 10 min under UV (400 W Xenon arc lamp with a filter WG 8, Schott Glass, Germany, transmitting light above 270 nm) while shaking at 0°C. Labelled mitochondria were pelleted at 8500×*g* for 10 min and excess of [^{3}H]AzHA was removed by three centrifugations in sucrose medium containing BSA followed by three further washings with BSA omitted. The last pellet was resuspended in 100 μl of isolation medium.

2.3. Protocol for photolabelling of the isolated uncoupling protein with [^{3}H]AzHA

Aliquots of 100 μg of UcP usually in 500 μl were incubated while shaking at 0°C first for 10 min with a competitor (or its solvent for control) and then [^{3}H]AzHA was added at given molar ratio to dimeric UcP (e.g. for 1:1 ratio: 9.6 μl of stock [^{3}H]AzHA containing just 2 nmol AzHA was added to 132 μg of UcP which is 2 nmol of 66 kDa) and incubated for another 10 min at 0°C. Samples were then illuminated in an ice bath for 10 min by UV lamp with the WG 8 filter as described above. Protocol when using [^{3}H]AzDA was principally the same.

2.4. Electrophoresis and autoradiography/fluorography of mitochondrial samples

Samples containing 200 or 300 μg of mitochondrial protein were mixed with the same volume of "4× concentrated buffer" (94 mM Tris-Cl, 30% glycerol, 6% SDS, 60 mg/l bromphenol blue, containing 2% mercaptoethanol), heated in boiling water bath for 10 min. Employing a Protean IIxi electrophoresis apparatus (Bio-Rad, USA), samples were separated on a Laemli SDS-PAGE (12% acrylamide; acrylamide/bisacrylamide 30:1, gel thickness 1 mm) at current density 15 mA/cm^2. After PAGE run (typically 5 h) the gel was bathed subsequently in 40% methanol for 30 min and in 10% trichloroacetic acid for 30 min. Staining was then performed by 0.05% Coomassie blue R in 50% methanol, 10% acetic acid for 2 h. The gel was destained by repeated bathing in 5% methanol, 15% acetic acid and then was photographed. After photographing, the gel was treated with a universal autoradiography enhancer ENTENSIFY and dried between plastic follies under vacuum. Dried gel with peeled-out top folly was mounted into the autoradiography steel cassette with an intensifying screen with a Kodak Scientific Imaging Film X-Omat, AR-5, and stored for typically about 5 days at −50°C with [^{3}H]AzHA and for several months with [^{3}H]AzDA. Film was developed in Kodak Dektol Developer for 25 min and fixed in Kodak Fixer for 5 min, both at 21°C. The resulting autoradiograms were scanned on Enhanced Laser Densitometer (LKB Bromma, Sweden) and photographed.

2.5. Electrophoresis and autoradiography/fluorography of UcP samples — determination of stoichiometry

UcP samples were run on a Laemli SDS-PAGE as described above mostly without previous washing of free label. In some experiments two identical gels were performed and one was stained with Coomassie blue and treated with an enhancer ENTENSIFY as described above (exposing films for about a week at −55°C), whereas the 32 kDa bands were excised from the second gel and electroeluted on a Six-Pac Electroeluter (Hoeffer Sci., USA) for 90 min at 90 V. Eluted UcP samples were estimated by modified Lowry procedure to evaluate protein content and by liquid scintillation counting to determine total bound [^{3}H]AzHA (in nmol). These determinations were taken for calculations of stoichiometry.

3. Results

3.1. Selective photolabelling of the uncoupling protein in BAT mitochondria with [^{3}H]AzHA

[^{3}H]AzHA incubated with BAT mitochondria at 1.5 nmol/mg protein and irradiated with UV for 10 min labelled only a few proteins in BAT mitochondria (around 7) as shown in the Fig. 1a,b, Lanes A. The photograph of the corresponding PAGE gel (Fig. 1a left, Lane A) clearly demonstrates how small a fraction of mitochondrial proteins was interacting with [^{3}H]AzHA. Among the few labelled protein bands apparent on the autoradiogram one can see the very prominent M_r-32 kDa band corresponding to UcP (Fig. 1a,b). In this region, approximately 24% of the total radioactivity was recovered. Beside UcP, the protein at 14, 16, 18, 45, and dublets

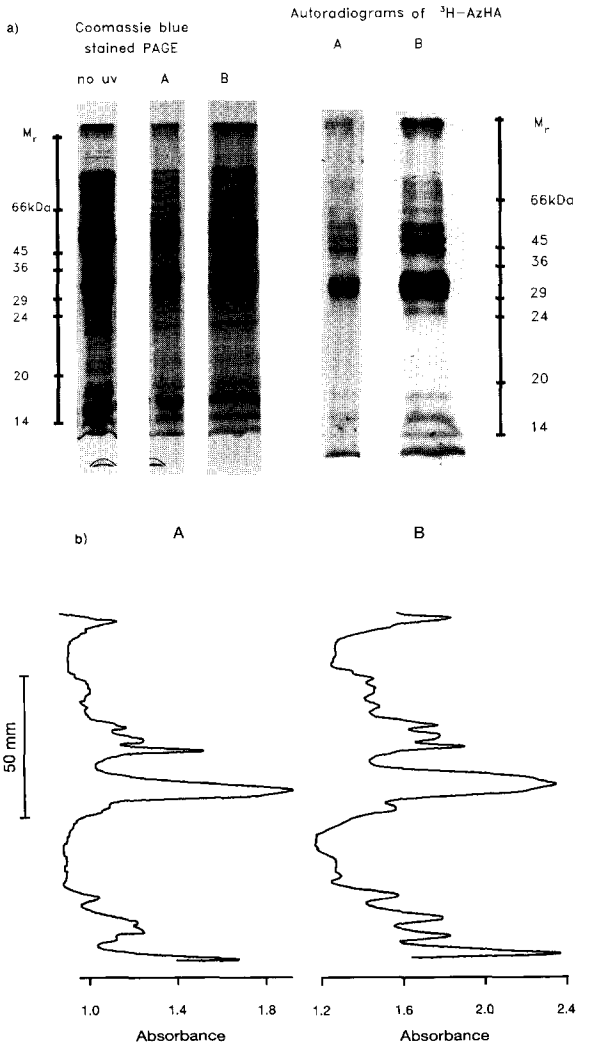

Fig. 1. Photolabelling of proteins of brown adipose tissue mitochondria with [^{3}H]AzHA. (a) Comparison of SDS-PAGE and autoradiograms. Left panel: photograph of SDS-PAGE; Right panel: autoradiogram of the corresponding lanes with accelerated screening of ^{3}H by enhancer. Lane A: 1.5 nmol [^{3}H]AzHA per mg mitochondrial protein, Lane B: 15 nmol [^{3}H]AzHA per mg mitochondrial protein. Scale in M_r was drawn according to positions of low molecular weight standards (Sigma). A separate sample of BAT mitochondria was run on the same SDS-PAGE for control ("no uv"). (b) Scan of autoradiograms of samples in Lane A, B. Absorbance on films was measured with Enhanced Laser Densitometer, as described in section 2.

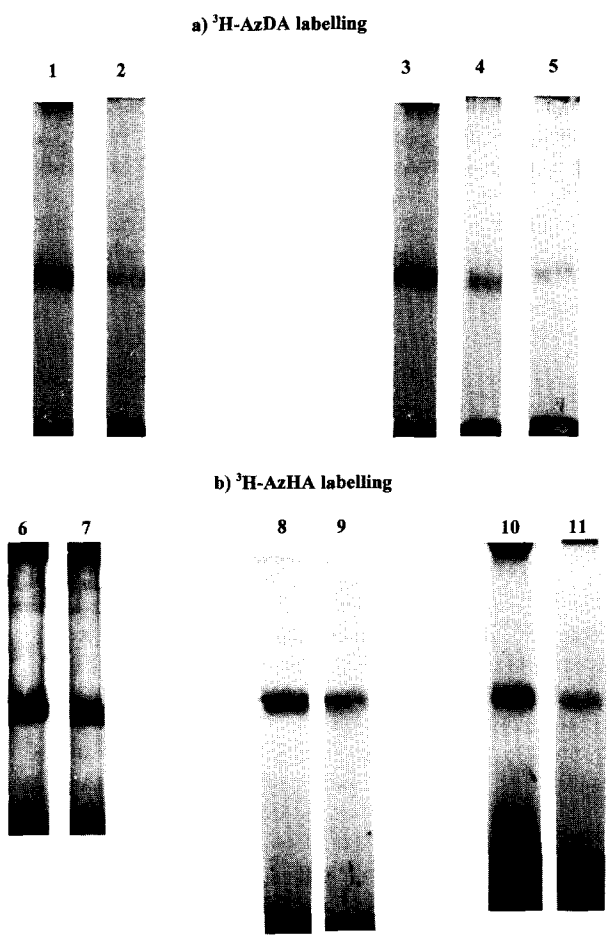

Fig. 2. Photolabelling of the isolated uncoupling protein with [^{3}H]AzDA and [^{3}H]AzHA. (a) [^{3}H]AzDA labelling and its prevention with stearic acid, hexanesulfonate and octadecanesulfonate. Autoradiograms are compared for control (Lane 1) and protection with stearic acid (Lane 2) or other control (Lane 3) and protection with hexanesulfonate (Lane 4) and octadecanesulfonate (Lane 5). Competitors were incubated with UcP for 10 min in the dark prior to [^{3}H]AzDA incubation and UV illumination. Molar ratio of [^{3}H]AzDA to dimeric UcP was 10:1. Film exposure lasted for 55 days. (b) [^{3}H]AzHA labelling and its prevention with undecanesulfonate, octadecanesulfonate and demonstration of no effect of methanesulfonate. Autoradiography of the pairs of control vs. competitor are shown for [^{3}H]AzHA photolabelling while protected with methanesulfonate (Lanes 6, 7), undecanesulfonate (Lanes 8,9) octadecanesulfonate (Lanes 10, 11). Competitors were incubated with UcP for 10 min in the dark prior to [^{3}H]AzHA incubation and UV illumination. Molar ratio of [^{3}H]AzHA to dimeric UcP was 2 (10 for Lanes 6, 7). Film exposures lasted for 5 days.

at 50 and 52 kDa were clearly labelled with [^{3}H]AzHA at the dose of 1.5 nmol per mg protein. In the region around 30 kDa more than a single UcP band may be labelled as seen from a broad diffuse spot on the autoradiogram in contrast to the structured multiple-band region on the SDS-PAGE. [^{3}H]AzHA labelling of these proteins is currently being studied in our laboratory.

At 10 times higher ratio (15.3 nmol AzHA/mg protein) a few other labelled protein bands appeared at 24, and at 60, 66, 70 kDa. Part of this can be considered as a nonspecific binding concomitant to every radioligand, another part reflects FA binding sites of lower affinity. Proteins at 60 to 70 kDa might be dimers of UcP and other anion carriers existing as monomers in the 30 kDa region. In the region of 32 kDa about 16% of the original radioactivity was recovered. Nevertheless, there is a remaining large group of mitochondrial proteins not interacting with [^{3}H]AzHA. Similar results were obtained with [^{3}H]azido derivative of dodecanoic acid (AzDa, not shown). Thus, we can conclude that the [^{3}H]AzHA-labelled mitochondrial proteins contain specific binding sites for fatty acids.

3.2. *[^{3}H]AzHA photolabelling of the isolated uncoupling protein*

When UcP was first isolated from BAT mitochondria and then photolabelled with [^{3}H]AzHA, the same 32 kDa band became visible on autoradiography (Fig. 2a,b). Similar results were obtained with shorter derivative [^{3}H]AzDA for which we had available also its nonradioactive form. Non radioactive AzDa protected more than 70% of the radioactive label (not shown). We have also found that BSA was photolabelled by both [^{3}H]AzHA and [^{3}H]AzDA under the same conditions (not shown). The amount of bound AzHA increased until the molar ratio of [^{3}H]AzHA to dimeric UcP (66 kDa) reached a value between 7 and 10. Further increase in this ratio up to 100:1 did not enhance [^{3}H]AzHA binding. Thus, changing the molar ratio of [^{3}H]AzHA:UcP we have evaluated a binding curve for this interaction (Fig. 3, upper trace) when bound AzHA was quantified by scanning autoradiograms and evaluating adsorbance of spots. Apparent K_d would correspond to a molar ratio of 1.5. In some experiments we have electroeluted labelled UcP from a gel and calculated stoichiometry from scintillation counting and protein estimations. At 10:1 molar ratio we were able to reach a maximum stoichiometry of 0.3 to 0.75 (4 determinations on 2 UcP preparations) at 10 min UV irradiation. Attempts to increase stoichiometry by longer UV exposure failed since a degradation of UcP occurred. This is probably because the absorption maximum of AzHA [13] is the same as the absorption maximum of tryptophans and tyrosines. In any case, the UV irradiation was the most critical step influencing stoichiometry of bound [^{3}H]AzHA. For example, exchanging filter,

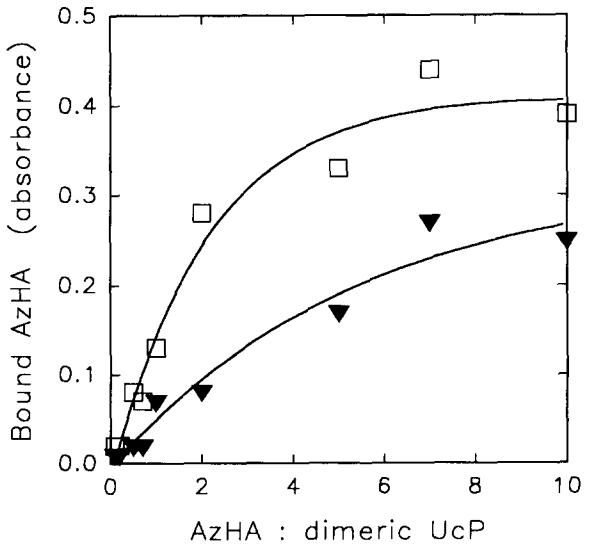

Fig. 3. Binding of [^{3}H]AzHA as a function of its molar ratio to dimeric UcP. (□) no competitor; (▼) octadecanesulfonate incubation prior to [^{3}H]AzHA. Binding was quantified as an intensity of spots in autoradiograms corrected for background (when required) while scanning autoradiograms. The films were exposed for 5 days. Experimental data were fitted using Marquart algorithm.

using other UV absorbing compounds, or decreasing time, all had a great effect on the amount of bound [^{3}H]AzHA.

3.3. Competition of fatty acids and alkylsulfonates with [^{3}H]AzHA

Selectivity of azido FA interaction suggests a specificity of a putative FA binding site on UcP. To probe its further properties, we studied competitive effects of FA and alkylsulfonates. The latter are closest analogues of FA which are known to be transport substrates of UcP [3,7]. When studied with 10:1 molar ratio of [^{3}H]AzDA (Fig. 2a), stearic acid added before incubation with [^{3}H]AzDA was able to prevent 77% of [^{3}H]AzDA label. Also alkylsulfonates protected against [^{3}H]AzDA photolabelling. Protection was more intense with the increasing chain-length reflecting the increasing affinity of UcP for more hydrophobic alkylsulfonates [3,7]. Thus, butanesulfonate prevented 30%, hexanesulfonate 47% (Fig. 2a), undecanesulfonate 62% and octadecanesulfonate protected 76% of the label (Fig. 2a). With [^{3}H]AzHA similar results were obtained. Thus stearic acid added in slight excess prior to [^{3}H]AzHA (10:1 molar ratio) lowered stoichiometry to 0.12, thus preventing 84% of [^{3}H]AzHA binding, and lauric acid to 0.09, preventing 88%. Prevention of [^{3}H]AzHA photolabelling was clearly pronounced when preincubation with 10-fold excess of octadecanesulfonate and undecanesulfonate was performed (Fig. 2b). They prevented 89% and 81% of [^{3}H]AzHA binding (from stoichiometry), respectively, or 71% and 64% when estimated from autoradiograms. Again, short chain alkylsulfonates were much less effective. Butanesulfonate prevented only by 22% and methanesulfonate showed no competition as apparent from autoradiography (Fig. 2b) as well as from stoichiometry. Compounds bearing two negative charges such as butylphosphonate, dodecanedioic acid, tetradecanedioic acid, or bipolar compounds such as 12-hydroxylauric and 12-aminolauric acid did not prevented [^{3}H]AzDA nor [^{3}H]AzHA binding. Also GDP did not prevent photolabelling with [^{3}H]AzHA. AzHA binding curve measured in the presence of octadecanesulfonate showed a typical shape expected for protection (Fig. 3, lower trace). However, in some experiments at molar ratios higher than 10 nonspecific binding resulted in a competitive effect becoming hidden (not shown), especially when films were overexposed.

4. Discussion

Fatty acids were recognized at the birth of bioenergetics as peculiar uncoupling-like agents [14]. Their action was later described as decoupling [15] and a novel point of view came with the fatty acid cycling hypothesis [3,9]. Any specific interaction with an integral mitochondrial membrane protein(s) requires the existence of a specific receptoric site in such a protein. If such sites exist on mitochondrial proteins for FA they should be able to interact with FA derivative such as AzHA and one should be able to identify them using photoaffinity labelling. This is indeed what we observed and we can consider that at least 5 to 10 proteins in BAT mitochondria are able to interact specifically with AzHA or AzDA. We may assume that this interaction proceeds also with physiologically abundant fatty acids. Identification of all these proteins is outside the scope of this paper, but the fact that such proteins exist is firmly established by our results.

Among FA-binding proteins of BAT mitochondria a most prominent labelled band corresponds to the 32 kDa monomeric UcP. Observed specificity of AzHA labelling is striking, especially when compared to the labelling with another hydrophobic label dicyclohexylcarbodiimide which binds to many more proteins in mitochondria [16]. Achieved stoichiometry was still below one, which is most probably a consequence of insufficient efficiency of photoreaction. Nevertheless, the low stoichiometry together with the high selectivity of photolabelling in mitochondria confirms the specificity of AzHA binding to UcP and supports the existence of the specific FA binding site on UcP. It excludes a nonspecific "coating" of the hydrophobic protein/membrane interface with the hydrophobic AzHA or other nonspecific effects.

A key factor for obtaining these results, however, was synthesis of highly tritiated azido fatty acid derivative [^{3}H]AzHA. The strategic step in its synthesis involved preparation of a mixture of unsaturated precursors, 16-aminohexadec-8-enoic and 16-aminohexadec-9-enoic acid. Subsequent catalytic hydrogenation proceeded with a substantial exchange into the aliphatic chain. From the obtained high specific activity of 4.8 TBq/mmol it has been derived that up to four tritium atoms were incorporated by this way.

Our results are also compatible with the fatty acid cycling mechanism proposed for UcP [3,9]. According to this "FA-protonophore model" [3] anionic FA are rather transport substrates of UcP. In this case UcP, by enabling FA uniport, allows for FA uncoupling circuit across the membrane similar to classic uncoupler cycling. In case of FA it is given by the natural ability of protonated FA to flip-flop across the membrane [17]. Our binding data support this mechanism in a sense that no tight FA binding was detected. Roughly estimated K_ds of 4 μM seems to be low enough, but considering a high partition coefficient of AzHA, it reflects rather high amount of FA present in the membrane. Therefore, it rather corresponds to a translocating binding site than to an allosteric regulatory site. Prevention of [^{3}H]AzHA photolabelling with other transport substrates of UcP such as undecanesulfonate [3] and hexanesulfonate [4,7] shows that the transport pathway (or a channel) is shared by these unipolar amphiphiles and FA. It is known that with increasing chain length of alkylsulfonates their translocation rates and affinity to UcP increase [7]. We now found that [^{3}H]AzHA binding is strongly prevented with long chain alkylsulfonates, lower competition is achieved with hexanesulfonate and weak with butanesulfonate but not with methanesulfonate (Fig. 2a,b). These results confirm previously reported functional competition of alkylsulfonates and FA measured in the reconstituted system [3,4,8] and the competition in binding with spin-labeled stearic acid [5].

Having stoichiometry of [^{3}H]AzHA photolabelling less that one and having selectivity towards UcP among hundreds of mitochondrial proteins and having competition of with [^{3}H]AzHA and [^{3}H]AzDA binding, one can conclude that we have targeted just the channel region of the UcP. This channel in UcP might be formed by the several transmembrane α-helices or by a single one and is probably exposed to lipid/protein interface [3,4]. We can speculate that such a channel might be formed by a single energy well [7], for example by a positive charge, located in the middle of membrane which could be accompanied by other amino acid residues to attract the hydrophobic parts of unipolar anions.

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