Photoaffinity labeling of mitochondrial proteins with 2-azido [^{32}P]palmitoyl CoA

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Abstract A long-chain fatty acyl CoA photolabel, 2-azido [^{32}P]palmitoyl CoA, was synthesized and its covalent interaction with mitochondrial membrane proteins examined. On binding of 2-azido [^{32}P]palmitoyl CoA to beef heart mitochondria, two polypeptides were primarily labeled, the 30 kDa ADP/ATP carrier and a 41 kDa protein of unknown identity. Carboxyatractyloside and palmitoyl CoA completely protected against labeling of the 30 kDa protein indicating that it was the ADP/ATP carrier. With inverted submitochondrial particles, only the 30 kDa polypeptide was labeled by 2-azido [^{32}P]palmitoyl CoA. The labeling was inhibited by bongkrekic acid and palmitoyl CoA but not carboxyatractyloside, providing evidence that the ADP/ATP carrier was covalently bound from the matrix side of the membrane. In brown adipose tissue mitochondria, 2-azido [^{32}P]palmitoyl CoA photolabeled the ADP/ATP carrier and the 32 kDa uncoupling protein with some minor labeling of 36 and 68 kDa polypeptides. The results indicated that this physiological photolabeling reagent with the azido group on the CoA portion of the molecule interacts like 2-azido ADP with nucleotide binding sites of a number of important enzymes in cell metabolism. Moreover, the evidence strongly supports the hypothesis that long chain fatty acyl CoA esters are natural ligands for key nucleotide binding proteins.

Key words: Mitochondrion; Photolabeling; Palmitoyl CoA; ADP/ATP Carrier; Uncoupling protein

1. Introduction

In addition to their substrate function, long chain fatty acyl CoA esters have been postulated to be regulatory ligands for a number of important enzymes [1–4]. In particular they have been observed to possess specific binding affinities for the nucleotide, carboxyatractyloside (CAT), and BKA receptor sites on the AAC in the inner mitochondrial membrane [5]. Somewhat similar properties of acyl CoA esters have been detected by their competition for the nucleotide binding site on the UCP [6,7]. In order to better characterize these site specific interactions, we have synthesized a physiological acyl CoA photolabeling reagent, 2-azido [^{32}P]palmitoyl CoA, which should prove useful for more definitive and extensive studies. The photolabeling azide as well as radioactive group is located on the adenine moiety of the CoA which competes for the nucleotide binding site(s) on the responsive proteins. Thus co-valent attachment occurs through the active portion of the molecule which bears a strong resemblance to 2-azido ADP. This communication presents some interesting results obtained from photolabeling experiments with isolated mitochondria and inverted submitochondrial particles.

2. Materials and methods

2.1. Synthesis of 2-azido [^{32}P]palmitoyl CoA

2-Azido AMP was synthesized by the procedure of Boulay et al. [8]. Activation of 2-azido AMP was carried out as described by Michelson [9]. D-Pantethine 4′4″-diphosphate was synthesized from D-pantethine as described by Shimizu [10] with a 40% yield. Bis(2-azido)dephospho CoA was synthesized from the two critical intermediates, activated 2-azido AMP and the tri-n-octylammonium salt of D-pantethine 4′4″diphosphate by the Michelson condensation reaction [9] with a yield of 25–35%.

Bis(2-azido)dephospho CoA was reduced to 2-azido dephospho CoA using sodium borohydride [11] with a 35% yield. The 2-azido dephospho CoA was then acylated by addition of the *N*-hydroxysuccinimide ester of palmitic acid as described by Al-Arif and Blecher [12].

The phosphorylation of 2 azido dephosphopalmitoyl CoA to 2-azido [^{32}P]palmitoyl CoA using [^{32}P]H_3PO_4 was carried out according to the method of Symons [13] as described by Boulay et al. [8]. Briefly, 25 nM Ci of [^{32}P]H_3PO_4 (as orthophosphate in dilute HCl) was diluted with 5 μl of 200 mM nonradioactive H_3PO_4 and freeze dried. Following the addition of 2 mg dry 2-azido dephosphopalmitoyl CoA and 10 μl triethylamine. The reaction mixture was incubated at 37°C for 30 min and immediately freeze dried.

The dry material containing the product was dissolved in 100 μl H_2O and the 2-azido palmitoyl CoA was identified by TLC on silica gel using a solvent system composed of butanol/acetic acid/H_2O (5:2:3, by volume). Phosphorylation was confirmed by running autoradiograms of the TLC plates containing the 2-azido [^{32}P]palmitoyl CoA. Finally, a preparative TLC plate was run on the remainder of the reaction mixture and the area of the silica gel corresponding to the migration of 2-azido [^{32}P]palmitoyl CoA was scraped from the plate and recovered by three extractions with 0.5 ml H_2O. The yield of 2-azido [^{32}P]palmitoyl CoA from 2-azido dephosphopalmitoyl CoA and [^{32}P]H_3PO_4 was approximately 10% and could be slightly improved by maintaining the pH in the lower alkaline range (7.5–8.0) to prevent hydrolysis of the thioester bond. The specific activity was approximately 2.8×10^8 cpm/μmol.

2.2. Preparation and photolabeling of mitochondrial fractions

Beef heart and BATM, and beef heart submitochondrial particles were prepared by standard procedures as previously described [5,6]. Photolabeling of mitochondria and inverted submitochondrial particles with 2-azido [^{32}P]palmitoyl CoA was carried out as previously published [14,15]. The photolabeled mitochondrial pellet was suspended a in minimal volume of sample buffer and SDS-PAGE was performed on 15% gels according to the Laemmli procedure [16]. The gels were stained by Coomassie blue, dried and autoradiographed at −70°C with Kodak XAR-2 X-ray film with intensifying screens.

2-Azido [^{32}P]palmitoyl CoA binding to mitochondria and ADP/ATP exchange experiments were performed as previously reported [14].

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Abbreviations: CAT, carboxyatractyloside; BKA, bongkrekic acid; AAC, ADP/ATP carrier; UCP, uncoupling protein; BATM, brown adipose tissue mitochondria; ACT-CoA, *N*-(3-iodo-4-azido-phenylpropionamido)cysteinyl-5-(2′-thiopyridyl)cysteine-coenzyme A; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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2.3. Materials

2-Chloroadenosine, hydrazine, $NaNO_2$, phosphorus oxychloride, D-panthetine, 2-cyanoethylphosphate, dicyclohexylcarbodiimide, chlorodiphenylphosphate, tributylamine, octylamine, sodium borohydride, triethylamine, palmitoyl CoA and carboxyatractyoside were purchased from Sigma Chemical Co. Trichloroacetonitrile was purchased from Aldrich Chemical Co. and [^{32}P]H_3PO_4 from New England Nuclear Co. Bongkrekic acid was a gift from Dr. W. Berends, Delft, The Netherlands. All other reagents were of the highest quality commercially available.

3. Results

The preliminary steps for the synthesis of 2-azido AMP have been previously described in the literature [8], whereas, we the more unique steps were developed in this laboratory to obtain the final product 2-azido [^{32}P]palmitoyl CoA from D-panthetine, 2-azido AMP and [^{32}P]H_3PO_4. The R_f of 2-azido palmitoyl CoA using two different solvent systems with TLC was very similar to that of commercial palmitoyl CoA (Fig. 1).

The photolabeling reagent incubated with beef heart mitochondria at a concentration of 10 μM inhibited ADP/ATP exchange by 90%. Binding studies were also carried out and the kDa was estimated to be 16 nM. These values are similar to those obtained with palmitoyl CoA [5] and indicate that the azide group does not interfere with effective binding to and inhibition of the carrier. The photolabeling results shown are from two separate experiments using preparations of beef heart and hamster brown adipose tissue mitochondria. Two other preparations of the photolabeled material gave similar results.

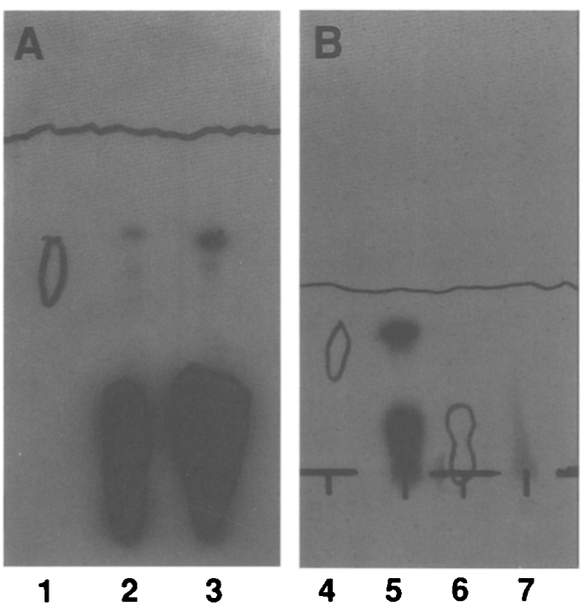

Fig. 1. (A) Autoradiogram of a silica gel TLC plate showing the migration of (1) palmitoyl CoA, non-labeled, open circle UV absorbing spot; (2) [^{32}Pi]palmitoyl CoA prepared by phosphorylation of dephosphopalmitoyl CoA; (3) 2-azido [^{32}P]palmitoyl CoA prepared by phosphorylation of 2-azido dephosphopalmitoyl CoA. The heavy band near the origin of lanes 2 and 3 includes free [^{32}Pi] and possibly some side product(s). The solvent system was butanol:acetic acid:H_2O (5:2:3, by volume). (B) Autoradiogram of a silica gel plate showing the migration of (4) palmitoyl CoA, non-labled, open circle, UV absorbing spot; (5) 2-azido [^{32}P]palmitoyl CoA (6) CoASH, non-labeled open circle, UV spotted; (7) an aliquot of an alkaline hydrolysate of 2-azido [^{32}P]palmitoyl CoA.

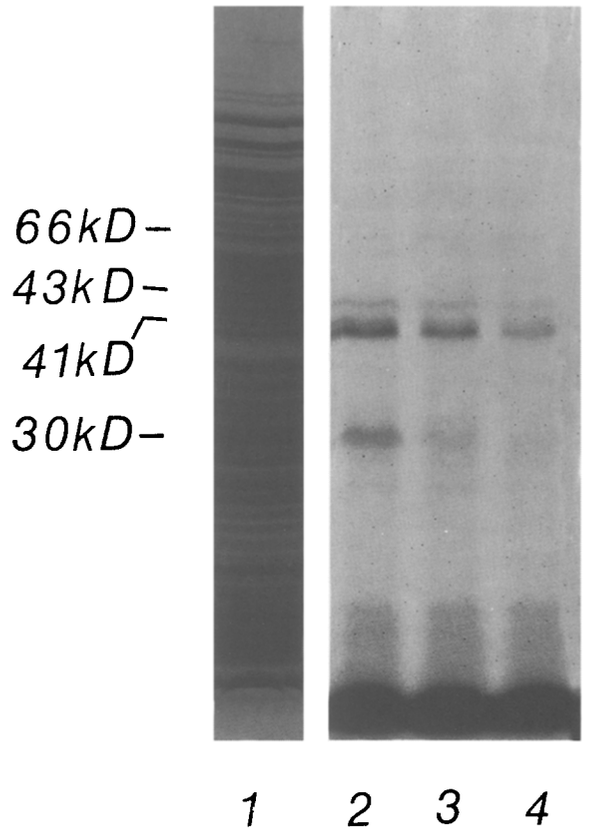

Fig. 2. Photolabeling of beef heart mitochondria by 2-azido [^{32}P]palmitoyl CoA. Mitochondria (200 μg protein) were incubated with or without CAT and palmitoyl CoA for 5 min followed by incubation with 2-azido [^{32}P]palmitoyl CoA (2.1×10^6 cpm) for 30 min at 4°C in the dark, the mixture was then diluted and immediately photolyzed for 5 s. The mitochondria were pelleted and solubilized in sample buffer for SDS-PAGE. Lane 1: SDS-PAGE pattern of labeled beef heart mitochondrial proteins. Lane 2: control autoradiogram. Lane 3: CAT, 25 μM. Lane 4: palmitoyl CoA, 15 μM.

3.1. Photolabeling of beef heart mitochondria

The photolabeling pattern of proteins from frozen intact beef heart mitochondria incubated with 2-azido [^{32}P]palmitoyl CoA is shown in Fig. 2. Lane 1 is an SDS gel of the photolabeled mitochondria. The other lanes represent the autoradiographs. Of primary interest is the photolabeled protein at 30 kDa (Lane 2) which corresponds to the AAC. Confirmation for the specific labeling of the AAC was obtained by protection against the photolabeling with CAT (Lane 3) and particularly with palmitoyl CoA (Lane 4), both of which bind at or near the nucleotide binding site. A protein at 41 kDa was also strongly labeled (Lane 2). The labeling pattern of this 41 kDa protein was not significantly effected by CAT but some protection was observed with palmitoyl CoA. A faintly visualized labeled protein was also noted at 43 kDa.

3.2. Photolabeling of beef heart submitochondrial particles

In Fig. 3, beef heart inverted submitochondrial particles incubated with 2-azido [^{32}P]palmitoyl CoA show labeling of only a 30 kDa protein, corresponding to the AAC which spans the inner mitochondrial membrane (Lane 2). CAT which binds to and inhibits the AAC only from the cytosolic side of the inner

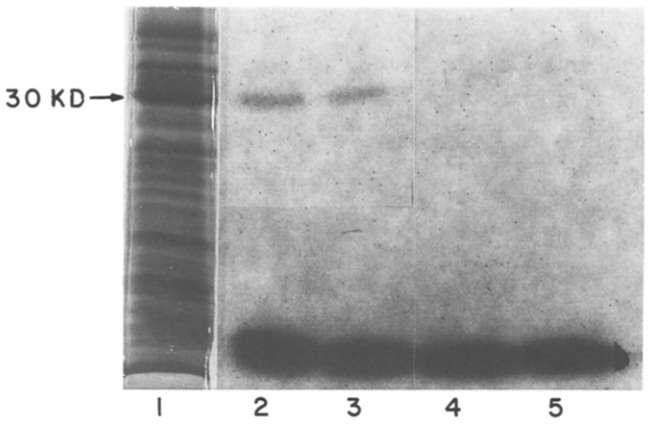

Fig. 3. Photolabeling of beef heart inverted submitochondrial particles. Incubation and labeling conditions were the same as in Fig. 2. The submitochondrial particles were sedimented by high speed centrifugation. Lane 1: SDS-PAGE pattern of inverted submitochondrial particles. Lane 2: control autoradiogram. Lane 3: CAT, 25 μM. Lane 4: BKA, 20 μM. Lane 5: palmitoyl CoA, 15 μM.

mitochondrial membrane [17–19] does not protect against photolabeling (Lane 3) and the band intensity is similar to that in Lane 2. BKA which binds to and inhibits the AAC only from the matrix side of the inner mitochondrial membrane [18] protects against photolabeling (Lane 4). Palmitoyl CoA which binds to and inhibits the AAC from both sides of the inner mitochondrial membrane [5,17] is as effective in protection against photolabeling with inverted submitochondrial particles as intact mitochondria (Lane 5). These results complement previously reported kinetic experiments [5,17]. In contrast to the results shown in Fig. 2 with intact mitochondria, photolabeling of other proteins was not observed with submitochondrial particles.

3.3. *Photolabeling of brown adipose tissue mitochondria*

A number of proteins were found to be photolabeled when BATM was incubated with 2-azido [^{32}P]palmitoyl CoA and photolyzed (Fig. 4). Proteins at 30 and 32 kDa (Lane 2) correspond to the AAC and UCP respectively as distinguished by the distinctive protection experiments as well as the molecular weights. Photolabeling was inhibited by specific ligands. When GDP was added to the incubation mixture to protect the UCP, only the 30 kDa AAC was labeled (Lane 3). When CAT was added to protect the AAC, only the UCP was photolabeled (Lane 4). Incubation with palmitoyl CoA which interacts with both proteins eliminates their photolabeling (Lane 5). As noted, additional higher molecular weight proteins were photolabeled, both in the absence and presence of these ligands.

4. Discussion

Various analogs of acyl CoA esters have been synthesized and used for photoaffinity labeling of acyl CoA dependent proteins [20–23]. Our own studies with ACT-CoA, an acyl CoA like photolabeling reagent, which reacted with the AAC prepared from beef heart mitochondria and the AAC and UCP from BATM have also been published [14,15]. These acyl CoA esters with azido, nitrophenyl or nitrophenoxyl photoactive groups, like ACT CoA, contain the photoactive group on the terminal end of the carbon chain rather than the CoA moiety of the compound. 2-Azido palmitoyl CoA overcomes this problem and represents a more physiological photolabeling reagent with which to assess the natural ligand binding and regulatory properties of acyl CoA esters.

The photolabeling experiments presented here clearly demonstrate specific covalent binding of 2-azido [^{32}P]palmitoyl CoA to the AAC and UCP which is protected against by appropriate ligands for the respective bovine heart and BAT mitochondrial proteins. Although more studies are indicated, an additional distinctive feature was that this photolabel could react with the AAC from both the cytosolic and matrix sides of the inner mitochondrial membrane. This important characteristic, which has not been detected with other photolabeling reagents including 2-azido ADP [24], supports previous results obtained by kinetic experiments with nucleotides and palmitoyl CoA on adenine nucleotide transport [5]. Thus, it has now been conclusively demonstrated that, whereas, there are asymmetric receptors for CAT and BKA on either side of the inner mitochondrial membrane, both binding sites recognize acyl CoA esters [25]. 2-Azido palmitoyl CoA would thus be a very useful photolabel for future structure/functional studies, particularly mapping the overlapping acyl CoA/nucleotide binding sites of the AAC in intact mitochondria and inverted submitochondrial particles. It can then be determined whether different residues of a single binding site, which are exposed as a result of conformational change, can bind nucleotides as well as acyl CoA esters in either state according to the gated pore model for translocation of nucleotides by the AAC [26].

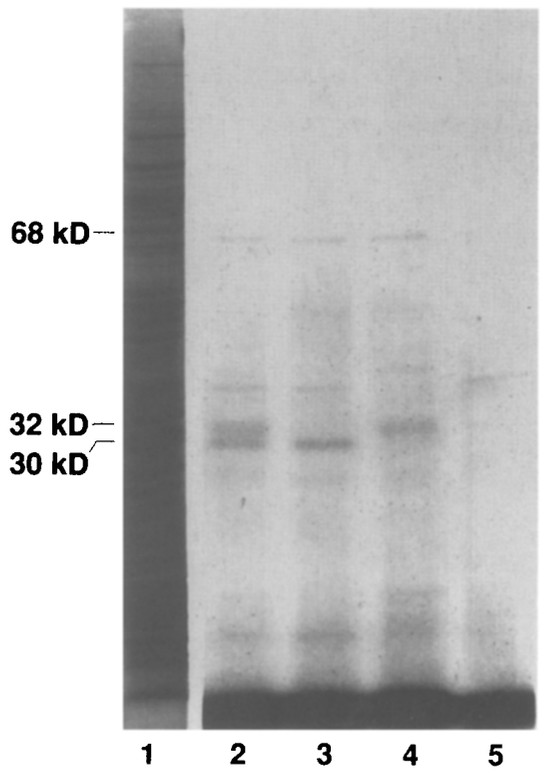

Fig. 4. Photolabeling of brown adipose tissue mitochondria by 2-azido [^{32}P]palmitoyl CoA. Incubation and labeling conditions same as in Fig. 2. Lane 1: SDS-PAGE pattern of labeled brown adipose tissue mitochondrial proteins. Lane 2: control autoradiogram. Lane 3: GDP, 25 μM. Lane 4: CAT, 100 μM. Lane 5: palmitoyl CoA, 15 μM.

Of additional interest was the observed distinct and intense labeling of a protein of approximately 41 kDa in isolated beef heart mitochondria which was somewhat protected against by palmitoyl CoA. A significant finding was the lack of photolabeling in inverted submitochondrial particles. Based on the estimated molecular weight and sidedness, the photolabeled protein could possibly be a subunit of creatinine phosphokinase, which is located on the outer side of the inner mitochondrial membrane and binds nucleotides. In BATM there is labeling of a 68 kDa protein which though not protected by palmitoyl CoA would correspond to the carnitine palmitoyl transferase enzyme. Further protection experiments and the use of two dimensional gels are required to clearly identify these proteins but the results indicate the versatility as well as specificity of the azido palmitoyl CoA.

A number of membrane bound and soluble proteins in various tissues, many of them nucleotide binding proteins, have now been recognized to be influenced by long chain acyl CoA esters. It should now be possible to determine any common features and homologies in the acyl CoA binding sites of these proteins. Moreover, a more detailed analysis of the acyl CoA binding site(s) on the AAC should provide valuable information not only on the mechanism of this important protein but also some insight on the physiological relevance of its regulation.

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