Photoaffinity labeling of the uncoupling protein UCP1 with retinoic acid: ubiquinone favors binding

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Abstract Retinoic acid is a potent activator of the uncoupling protein-1 (UCP1) both at the gene and mitochondrial level. Irradiation with ultraviolet light can be used to directly photolabel proteins with retinoic acid. The procedure has been applied to investigate its interaction with UCP1 isolated from brown adipose tissue mitochondria. All-*trans*-retinoic acid binds to UCP1 with high affinity and the labeling is only partially protected by guanosine diphosphate. Ubiquinone (UQ) has been described to be an obligatory cofactor for uncoupling protein function and we demonstrate that it greatly increases the affinity of UCP1 for retinoic acid. Data support the notion of a direct interaction between UQ and retinoic acid.

Key words: Uncoupling protein; Retinoic acid; Photoaffinity labeling; Ubiquinone

1. Introduction

The uncoupling proteins are mitochondrial transporters whose biological function is the dissipation of the proton electrochemical potential gradient generated by the respiratory chain (reviewed in [1,2]). Genes coding for uncoupling proteins are widely distributed not only among animals but also in plants [1]. A regulated decrease of the efficiency of oxidative phosphorylation has been proposed to serve several biological functions like thermogenesis, maintenance of the redox balance or reduction in the generation of reactive oxygen species (reviewed in [2,3]).

UCP1 was the first uncoupling protein described and therefore it is the best characterized. It is only present in brown adipose tissue and it clearly has a thermogenic role (reviewed in [4]). The physiological regulation of UCP1 is well established (reviewed in [4,5]). Purine nucleotides maintain the protein inhibited by binding to a site from the cytosolic side of the membrane. Noradrenaline stimulation of the brown adipocyte initiates a lipolytic cascade and the fatty acids released serve two functions: they are substrates for respiration and activators of UCP1. Fatty acids over-ride the nucleotide inhibition to activate proton transport and thus initiate thermogenesis. We have described the activation of UCP1 by all-*trans*-retinoic acid (RA) [6], a new regulatory pathway possibly of physiological significance since RA is a potent activator of the transcription of the *ucp1* gene [7,8].

Ubiquinone (UQ) has recently been described as an essential cofactor for the activity of UCP1 [9]. The authors reported that when UCP1 is expressed recombinantly in *Escherichia coli*, the fatty acid dependent proton transport activity can only be reconstituted if UQ is present. Subsequently, the same group described that the uncoupling proteins UCP2 and UCP3 could also be reconstituted functionally if the same conditions were met [10]. There is, however, no consensus on the UQ requirement since other researchers have reported the reconstitution, from inclusion bodies, of a nucleotide-sensitive activity without UQ [11,12].

Photoaffinity labeling is an approach widely used to identify protein regions involved in ligand binding. The presence in RA of the conjugated polyene chain allows its direct use to photolabel proteins without the introduction of other photoreactive groups [13]. In this report, we have used the published methodology to investigate the binding of RA to UCP1 and the relationship between binding of this activator and other ligands of the UCP1. We demonstrate that RA can be bound covalently to UCP1 and that its binding is partially inhibited by nucleotides but, interestingly, greatly enhanced by UQ.

2. Materials and methods

2.1. Purification of UCP1

UCP1 was purified from the brown adipose tissue of cold-adapted hamsters essentially as previously described [14]. Intrinsic membrane proteins were extracted with 4% Triton X-100, using 1.28 g Triton X-100 per gram of mitochondrial protein. UCP1 was purified by conventional hydroxyapatite chromatography but the sucrose gradient step was omitted. The protein was concentrated to 1 mg/ml and the Triton X-100 content was lowered to around 1.5%, removing the excess detergent with Bio-Beads. Protein concentration was determined by the Lowry method in the presence of sodium dodecyl sulfate (SDS) and Triton X-100, using bovine serum albumin (BSA) as standard. Hydroxyapatite and Bio-Beads SM-2 were from Bio-Rad Laboratories.

2.2. Labeling of UCP1 with retinoic acid

UCP1 was photolabeled with all-*trans*-[11,12-^{3}H]retinoic acid by irradiating with UV light. UCP1 (0.25 mg/ml) was incubated in a buffer containing 20 mM Na_2SO_4, 0.16 mM EDTA, 20 mM Mops pH 6.7 and 25 μM butylated hydroxytoluene (a free radical scavenger). Concentration of all-*trans*-[^{3}H]retinoic acid was 60 μM and 10 μCi/ml and represents a 8:1 molar ratio to UCP1. Samples were irradiated, under continuous stirring, at 360 nm (20 nm bandwidth) with a 150 W Oriel lamp and a photon flux density of 200 μmol/m^2 s. A control sample was taken before each irradiation to determine the contaminating label that was not covalently bound. Free RA was removed by washing with acetone and *tert*-butyl methyl ether. Protein

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Abbreviations: RA, all-*trans*-retinoic acid; UQ, ubiquinone

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samples were precipitated with acetone (4:1) and kept overnight at −20°C. Samples were centrifuged and the resulting pellets were subjected to a wash with butyl methyl ether and further wash with acetone. The final pellets were dissolved in 100 μl of 10% SDS by warming at 60°C for 5 min. Scintillation cocktail was then added and the radioactivity determined. Retinoic acid was added as a methanolic solution while UQ was dissolved in acetone. Guanosine diphosphate (GDP), UQ, palmitic acid, retinoic acid, BSA and Triton X-100 were from Sigma Chemical Co.; all-*trans*-[11,12-^{3}H]retinoic acid from NEN Life Sciences Products (Boston, MA, USA). All other reagents were of the highest purity commercially available.

2.3. Nucleotide binding

Nucleotide binding to isolated UCP1 was determined essentially as previously described [15]. Assay conditions were set as close as possible to the labeling experiments. The binding buffer contained 20 mM Na_2SO_4, 0.16 mM EDTA, 20 mM Mops pH 6.7, 25 μM butylated hydroxytoluene, [^{14}C]sucrose (0.18 μCi/ml) and varying concentrations of [^{3}H]GDP (0.68 μCi/ml). UCP1 concentration was lowered to 0.08 mg/ml (2.4 μM) and the estimated Triton X-100 content was 0.17%. Binding assays were performed in the presence and absence of 19 μM RA to maintain an 8:1 molar ratio. [8-^{3}H]GDP was from Amersham Pharmacia Biotech and [^{14}C(U)]sucrose from NEN Life Sciences Products.

3. Results and discussion

We have previously reported that proton transport through UCP1 is activated by RA in a nucleotide-sensitive manner [6]. This regulation is probably of physiological relevance since the activation by RA of *ucp1* gene transcription has been demonstrated [7,8]. Moreover, the RA concentrations required to activate proton transport and gene transcription are within the levels found in brown adipose tissue [16]. The activation kinetics have demonstrated that the affinity of UCP1 for RA is higher than that observed for palmitate, a fatty acid commonly used to activate the protein.

Bernstein et al. [13] have previously shown that all-*trans*-[^{3}H]retinoic acid can be used to photoaffinity-label retinoic acid-binding proteins. The presence of the photoreactive polyene structure in this natural ligand makes it an ideal probe to investigate its interaction with the binding site. The methodology has subsequently been used by different groups to characterize retinoid binding sites and even to identify new retinoid binding proteins [17–19]. Despite the reported low labeling efficiency of the process [13,19] and the presence of detergent, the above-mentioned high-affinity of UCP1 for RA was a stimulation for the project. Fig. 1 shows a time course of the labeling of UCP1 with RA. We used a relatively low RA/protein ratio (8:1) to minimize the non-specific labeling. Higher ratios only marginally increased the labeling (data not shown). Fig. 1 reveals that 80% of the labeling occurs within the first hour and it is nearly complete after 2 h, probably due to the photodegradation of the ligand. Extensive washing with acetone and ether should ensure an efficient removal of the unbound RA and we have found that the contaminating unbound radioactivity represents around 10% of the counts found after 2 h incubation. Finally, the radioactivity bound to the protein could be displaced with cold retinoic acid. Thus, when parallel experiments were performed with constant label but raising the ratio cold-RA/protein from 5:1 to 50:1, the bound radioactivity decreased by 72%.

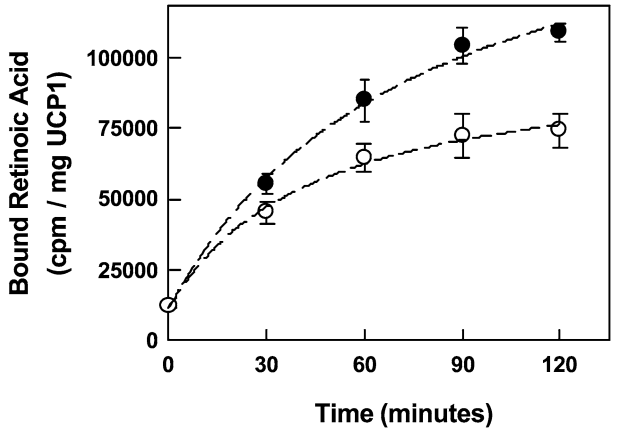

Fig. 1. Time course of the photoaffinity labeling of UCP1 with all-*trans*-[11,12-^{3}H]retinoic acid. UCP1 isolated from brown adipose tissue was irradiated with UV light (360 nm) in the presence of a eight-fold molar excess of RA. Experiments were performed either in the presence (empty circles) or absence (closed circles) of GDP 1 mM. Data points represent the mean ± S.E.M. of three independent experiments done in duplicate. Zero data point represents the contaminating radioactivity that is non-covalently bound.

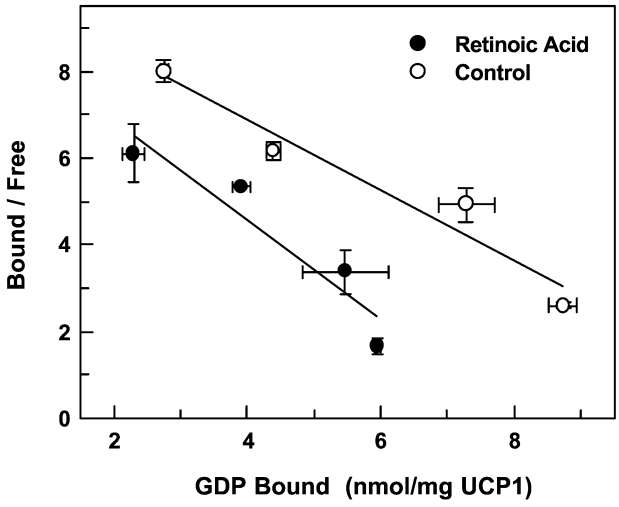

Fig. 2. Scatchard analysis of the effect of retinoic acid on the binding of [^{3}H]GDP to UCP1. Nucleotide binding experiments were performed either in the presence (closed circles) or absence (empty circles) of 8:1 RA. GDP concentrations ranged from 0.5 to 4 μM. The estimated B_{max} was 11.8 ± 0.9 nmol/mg for the control and 7.4 ± 0.5 nmol/mg in the presence of RA. K_d was 1.1 ± 0.2 μM in the control and 0.7 ± 0.2 μM when RA was present.

Since GDP inhibits the proton conductance increase mediated by RA [6], we tested its effect on the labeling. GDP also inhibits UCP1 labeling with RA although the protection is only partial (Fig. 1). Kinetics demonstrate that during the initial stages, GDP is nearly unable to prevent binding of RA to UCP1. In fact, when nucleotide binding experiments were performed in the presence of RA (8:1) marked inhibition of GDP binding was observed (Fig. 2). At a later stage, when an increasing proportion of the unbound RA is probably a poor ligand due to its photodegradation, GDP can efficiently compete and nearly blocks further labeling (Fig. 1). These results re-emphasize the high affinity of UCP1 for RA that can even prevent nucleotide binding.

UQ has been described to be an obligatory cofactor for the activity of the uncoupling proteins [9,10]. Since the structure of UQ resembles somehow the structure of RA (amphipatic molecules with an isoprenoid chain) we tested the influence of UQ on RA binding and we found that it markedly increases

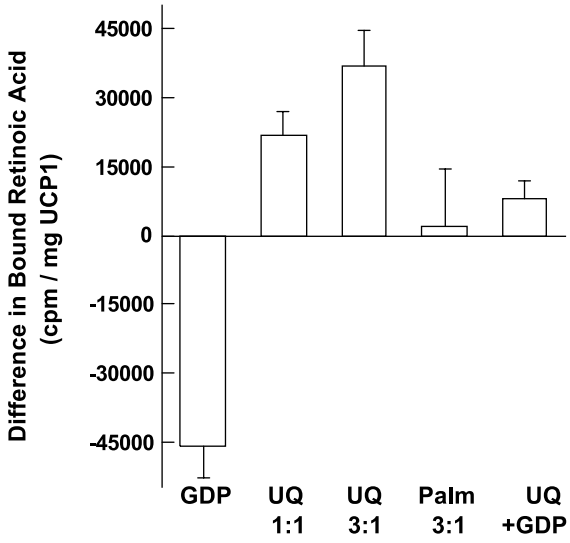

Fig. 3. Effect of GDP, UQ and palmitate (Palm) on the labeling of UCP1 with all-*trans*-[11,12-^{3}H]retinoic acid. Bars represent the mean ± S.E.M. of the difference in bound retinoic acid in the presence and absence of each effector. Three to five paired experiments were performed for each condition. In all cases, bound radioactivity was determined after irradiation for 2 h. Concentration of GDP was 1 mM. UQ was present at either 1:1 or 3:1 molar ratio to RA. Palmitate was 3:1 to RA. Concentration of RA was 60 μM and represents a 8:1 molar ratio to UCP1.

the labeling (Fig. 3). The UQ content of UCP1 isolated from brown fat mitochondria in Triton X-100 has been estimated to be 3 mol/mol of UCP1 [9]. Under our experimental conditions, it is to be expected that removal of the detergent with Bio-Beads will also eliminate part of the UQ. We have used two different concentrations of UQ, either equimolar to RA or a 3:1 ratio. The corresponding ratios to UCP1 were 8:1 and 24:1. Labeling is higher at the highest ratio, where the increase is 33% (Fig. 3). The labeling in the presence of UQ is still nucleotide-sensitive although the decrease in counts is lower than in its absence and thus reinforces the idea that UQ increases the affinity for RA. To test if other amphiphiles had the same effect as UQ, we investigated if palmitate (3:1 to RA) influenced the labeling, but no significant differences were found (Fig. 3). We could also have expected a lower labeling due to a competition between palmitate and RA because we have previously reported that both palmitate and RA act at the same site [6]. However, since the affinity for RA is much higher, the lack of effect can be easily understood.

Klingenberg and coworkers [9,10] have suggested that the molecular role of UQ in UCP1 function was intimately linked to the role of the fatty acids. In the present paper we demonstrate that the affinity for RA, another important regulator of UCP1, is greatly increased by UQ and strengthens the impression that there must exist a direct interaction between these two UCP ligands. The methodology presented in this report can be used to photoaffinity-label UCP1 with the aim of the identification of the regions involved in the activation of UCP1 by retinoids. Work is currently underway to identify specific amino acids involved in the formation of the retinoid binding pocket.

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