CHEMICAL RADIOLABELING OF CARBOXYATRACTYLOSIDE BY [^{14}C]ACETIC ANHYDRIDE

Binding properties of [^{14}C]acetylcarboxyatractyloside to the mitochondrial ADP/ATP carrier

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1. Introduction

Progress in knowledge of the molecular properties of the mitochondrial ADP/ATP carrier has benefited from the use of specific inhibitors, atractyloside, carboxyatractyloside and bongkrekic acid [1]. Binding and inhibition data led to the conclusion that AT and CAT attack the ADP/ATP carrier from the outside of the mitochondrial membrane and BA from the inside. Assuming that the ADP/ATP carrier is an intrinsic protein functioning as a channel and spanning the inner mitochondrial membrane, it has been postulated that the outer face of this channel binds atractyloside and the inner face bongkrekic acid [1]. The first binding studies dealing with mitochondria or submitochondrial particles were done with biosynthetically labeled inhibitors [2–4]. However, the specific radioactivity of biosynthetically labeled AT, CAT and BA was too low to permit further studies on the isolated carrier. For this purpose, chemically labeled AT and BA were prepared [5,6]. AT was chemically labeled either by replacement of one of the two carboxyl groups in CAT by ^{3}H, or by oxidation of the primary alcohol of the glucose disulfate moiety followed by reduction with [^{3}H] borohydride. The latter method of labeling appeared feasible for the labeling of CAT; however the yield of the reaction was always very low, which may be explained by the different configurations of the AT and CAT molecules [3].

Abbreviations: Ac-CAT, acetylcarboxyatractyloside; CAT, carboxyatractyloside; AT, atractyloside: BA, bongkrekic acid; TAB buffer, triethylamine adjusted to pH 8.7 with ammonium bicarbonate; TLC, thin-layer chromatography

We report here the synthesis and biological properties of a radiolabeled derivative of CAT obtained by acetylation of the primary alcohol of CAT with radiolabeled acetic anhydride. Acetylcarboxyatractyloside (fig.1) acted biologically as a substitute of CAT; it exhibited virtually the same inhibitory and binding properties as CAT, i.e., it behaved in saline medium as in irreversible inhibitor of ADP/ATP transport in contrast to AT whose inhibition is removed by excess ADP. Its binding affinity was close to that of CAT (K_d 2–10 nM). Ac-CAT pre-bound to mitochondria was readily displaced by CAT, but not by AT. The Ac-CAT–ADP/ATP carrier complex was stable after solubilisation. We also investigated the question of mutual exclusion of CAT and BA for binding to the mitochondrial ADP/ATP carrier in double labeling experiments based on the use of [^{3}H]BA and [^{14}C]Ac-CAT. The results are consistent with the view that the ADP/ATP carrier possesses two separate interacting binding sites for AT (or CAT) and for BA.

2. Materials and methods

2.1. *Materials*

Carboxyatractyloside was purchased from Boehringer, and atractyloside from Sigma. Bongkrekic acid was prepared as in [6]. [^{3}H]- and [^{14}C]acetic anhydride were obtained from the 'Commissariat à l'Energie Atomique', Saclay.

2.2. *Synthesis and purification of Ac-CAT*

Triethylamine (160 μl) was added to 600 μl of an aqueous solution of CAT (30 mg/ml) in a 2 ml

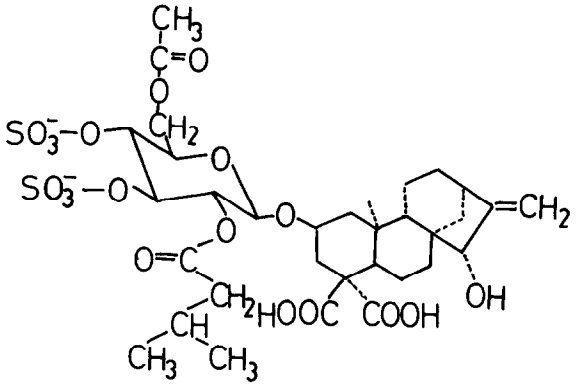

Fig.1. Structure of Ac-CAT.

Reactivial (Pierce) equiped with a triangular magnetic bar. Twelve successive additions of 2 μl of radioactive acetic anhydride were made with vigorous stirring at room temperature. There was a minimal period of 4 min between successive additions of the anhydride. The reaction mixture was then dried under vacuum and washed 5 times with methanol. The residue was dissolved in a minimum volume of methanol and streaked on 8 silica gel analytical K6 Whatman TLC plates (20 × 20 cm). The plates were developed with chloroform, methanol, acetic acid and water (55:25:6:4). Two narrow bands of 0.5 cm width on the lateral edges of the plates were cut out and the localisation of labeled Ac-CAT was revealed by staining with 1% vanillin in pure H_2SO_4 and by autoradiography. The unstained portion of the plates corresponding to Ac-CAT (R_F 0.28) was scraped off, and the powder was extracted with methanol. After evaporation to dryness, the residue was taken up in 2 ml 20 mM TAB buffer, diluted with water for conductivity adjustment, and applied to a DE-52 column (Whatman) (1.5 × 20 cm) equilibrated with 20 mM TAB. The column was washed with 100 ml 20 mM TAB and the radioactive Ac-CAT was eluted with 100 mM TAB buffer. The radioactive eluate was brought to pH 6 with 5 N acetic acid, then lyophilised. Ac-CAT was kept at −20°C as a methanolic solution. It was stable for months. When purified radiolabeled Ac-CAT was chromatographied in the silica gel system (fig.2) or on reversed phase TLC plates (KC_{18} Whatman) developed with a mixture made of methanol, water, acetic acid (50:50:0.6) (not shown), only one spot was revealed either by vanillin staining or by autoradiography, corresponding to the monoacetylated derivative of CAT (see below). It must be noted that higher concentrations of acetic anhydride led to the formation and accumulation of diacetyl CAT.

Specific activity of [^{3}H]- or [^{14}C]acetic anhydride was determinated by acetylation of cyclic AMP in aqueous medium in the presence of triethylamine, exactly as in [7,8]. The acetylated cyclic AMP was further purified by two successive TLC on cellulose developed with *n*-butanol, acetic acid and water (12:3:5) as in [9]. Aliquots were withdrawn for spectroscopic measurement and radioactivity estimation. The concentration was calculated from the A_{256} max (ϵ = 14 500 $M^{-1} . cm^{-1}$).

On the basis of the calculated specific radioactivity, the yield of radioactive Ac-CAT synthesis was approximated to 10%; specific radioactivities were 8.10^6 dpm/μmol and 10^8 dpm/μmol for [^{14}C]Ac-CAT and [^{3}H]Ac-CAT, respectively.

2.3. *Mass spectroscopy*

Mass spectra of CAT and Ac-CAT were taken with a Kratos MS50 apparatus. Samples (1–2 μg) were introduced into the ion source, heated at 200°C, working in electron impact at 70 eV. CAT and ac-CAT were treated for 1 h at 100°C with closed caps with pyridine, hexamethyldisilazane and trimethylchlorosilane (4:2:1) [10]. The reagents were evaporated and the residue taken up in chloroform. Peaks at mass to charge (*m/e*) ratios of 433, 361 and 289 were obtained

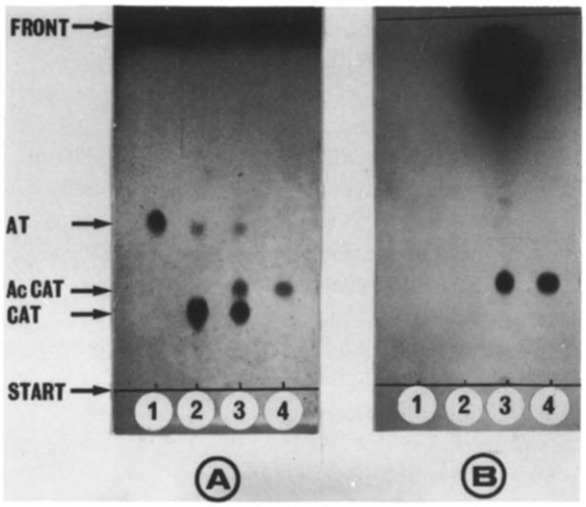

Fig.2. TLC of Ac-CAT or other AT derivatives on silica gel K6 Whatman plate. The plate was developed with chloroform, methanol, acetic acid and water (55:25:6:4, by vol.). (A) Vanillin staining: 1- AT; 2- CAT; 3- crude preparation of [1-^{14}C]Ac-CAT; 4- purified [^{14}C]Ac-CAT. (B) Autoradiograph.

for the sugar moiety of Ac-CAT (disilylated sugar moiety) and at 463, 391 and 319 for CAT (trisilylated sugar moiety) in accordance with [10]. The latter peaks were totally absent in the case of silylated Ac-CAT, indicating that acetylation of the primary alcohol function of the sugar moiety was complete. The steroid moiety showed peaks at 447 and 375, no peak was detected at 477 or 357, indicating that no acetylation had occurred on the steroid moiety.

2.4. *Subcellular preparations*

Rat heart mitochondria, rat liver mitochondria and beef heart mitochondria were prepared as in [11–13], respectively.

2.5. *Transport and binding assays*

The rate of [^{14}C]ADP transport was measured at 0°C by the direct exchange procedure [1], using a saline medium made of 120 mM KCl, 10 mM MES, 1 mM EDTA (pH 6.5). The reaction was started by addition of [^{14}C]ADP and terminated with 10 μM CAT followed by centrifugation. Binding assays were performed by incubating inhibitors in 5 ml above saline medium with mitochondria (1 mg protein) for 30 min at 0°C. After centrifugation, the pellets were washed twice with ice cold medium and solubilized with 5% Triton X-100, 0.5 M NaCl. Radioactivity was estimated by liquid scintillation.

3. Results

3.1. *Binding of [^{14}C]Ac-CAT to beef heart mitochondria*

The binding curve in fig.3 shows the presence in beef heart mitochondria of saturable binding sites for [^{14}C]Ac-CAT. The n. binding sites was 1.2 mol/mg protein with K_d 10 nM. Binding assays carried out with freshly prepared mitochondria from rat heart or liver yielded K_d 2–5 nM. Under the conditions described (saline medium), the binding of [^{14}C]-Ac-CAT was not modified by ADP ≤ 50 μM; at 100 μM ADP, a slight competitive effect was observed. The binding curve of [^{14}C]Ac-CAT was sigmoidal, and the Scatchard plot of the binding was characterized by a marked curvature. By all these binding properties, Ac-CAT mimicked CAT.

3.2. *Correlation between binding of [^{14}C]Ac-CAT and inhibition of [^{3}H]ADP transport by Ac-CAT*

[^{3}H]ADP transport measurements were done with

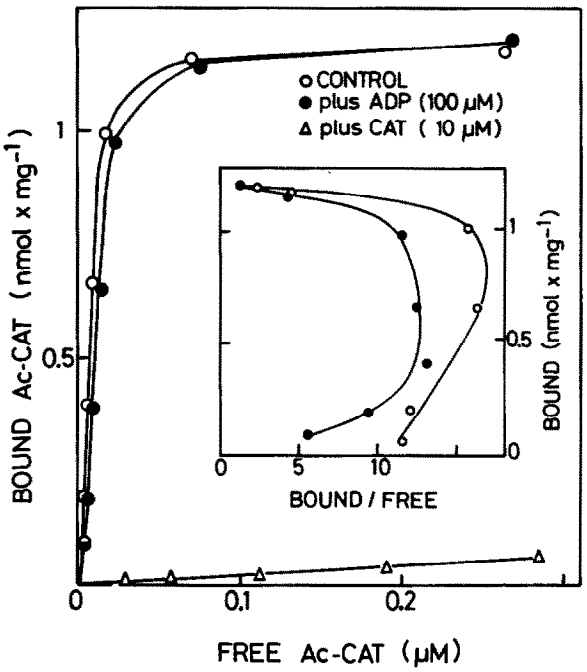

Fig.3. Binding of [^{14}C]Ac-CAT to beef heart mitochondria. Conditions are in section 2. Scatchard plots of [^{14}C]Ac-CAT in the presence and absence of 100 μM ADP are presented in the inset.

a saturating concentration of [^{3}H]ADP (50 μM). The effect of increasing concentrations of [^{14}C]Ac-CAT on [^{3}H]ADP transport was measured. The amount of bound [^{14}C]Ac-CAT was also determined. Binding of [^{14}C]Ac-CAT and inhibition of [^{3}H]ADP transport by [^{14}C]Ac-CAT were normalized in terms of percentages of binding and transport, respectively. As shown in fig.4, an excellent correlation exists between the binding of [^{14}C]Ac-CAT to mitochondria and the inhibition of ADP transport, indicating that Ac-CAT binds at specific sites on the ADP/ATP carrier.

3.3. *Solubilization of the Ac-CAT/ADP–ATP carrier complex*

Beef heart mitochondria were incubated with [^{14}C]Ac-CAT for 30 min at 0°C. Incubation was terminated by centrifugation. The pellets were solubilized by 4% Triton X-100, 0.5 M NaCl, and 10 mM MOPS (pH 7.2) and the ADP/ATP carrier protein was purified on a hydroxyapatite column equilibrated with 0.1% Triton X-100, 0.1 M NaCl and 10 mM MOPS (pH 7.2) [14]. The excluded peak was concentrated 4 fold by pressure dialysis on PM 10 Amicon membrane and applied to an Ultrogel type

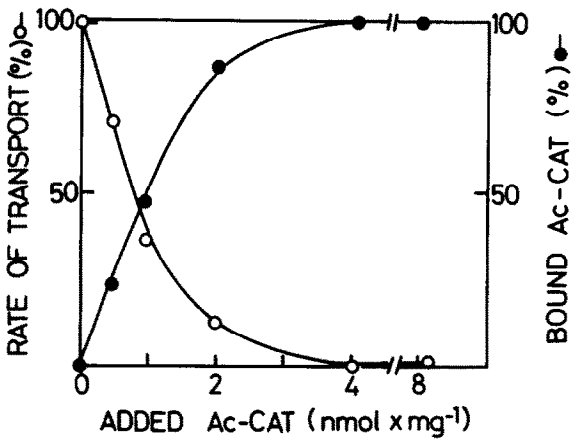

Fig.4. Correlation between binding of [^{14}C]Ac-CAT and inhibition of [^{3}H]ADP transport. Rat liver mitochondria (1 mg/ml in standard saline medium) were preincubated with increasing concentrations of [^{14}C]Ac-CAT for 30 min at 0°C. Inhibitor binding was determined on small aliquots after centrifugation as in section 2. The saturation plateau was at 0.2 nmol Ac-CAT/mg protein. Direct [^{3}H]ADP exchange was measured with 50 μM [^{3}H]ADP as in section 2. The rate of transport at 0°C in the absence of Ac-CAT was 2 nmol . min^{-1} . mg protein^{-1}.

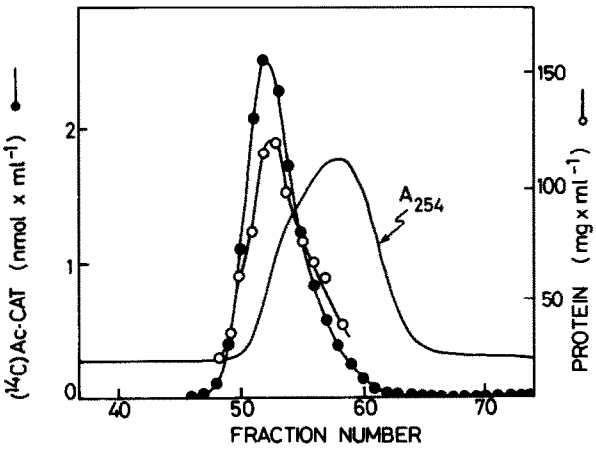

Fig.5. Gel filtration of the ADP/ATP carrier–Ac-CAT complex. The ADP/ATP carrier–[^{14}C]Ac-CAT complex was partially purified from beef heart mitochondria preincubated with [^{14}C]Ac-CAT by hydroxyapatite chromatography (section 3.3). Partially purified carrier protein (1 mg) was applied to an Ultrogel (AcA34) column (50 × 1.5 cm) equilibrated with 0.1% Triton X-100, 0.1 M NaCl, 10 mM MOPS (pH 7.2). Fractions of 1 ml were collected and processed for determination of protein and radioactivity. Triton concentration was monitored by measurement of A_{254}.

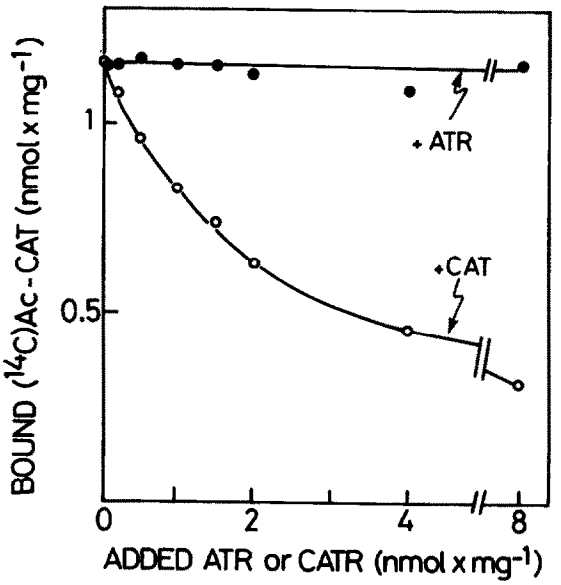

Fig.6. Competition between [^{14}C]Ac-CAT, CAT and AT for binding to mitochondria. [^{14}C]Ac-CAT (2 nmol) was added to beef heart mitochondria (1 mg protein) in 5 ml of standard saline medium) (see section 2) with increasing concentrations of CAT or AT. After 30 min incubation at 0°C, the suspension was centrifuged and the mitochondrial pellet was processed for radioactivity counting.

AcA34 column. The major protein peak and the radioactivity peak eluted from Ultrogel were superimposed (fig.5). A control experiment without mitochondria showed that free [^{14}C]Ac–CAT is eluted after the Triton X-100 peak; as no free [^{14}C]Ac-CAT was found in hydroxyapatite chromatography in the presence of mitochondria, it can be concluded that all the added [^{14}C]Ac-CAT had bound to the carrier protein indicating that the Ac-CAT carrier protein complex is stable. In this respect, the behaviour of [^{14}C]Ac-CAT is similar to that of biosynthetically labeled [^{35}S]CAT [15].

3.4. *Interaction between Ac-CAT and CAT or AT for binding to mitochondria*

[^{14}C]Ac-CAT was added to heart mitochondria at 0.4 μM final conc. with increasing concentrations of AT and CAT. As shown in fig.6 CAT, but not AT, competed with the binding of [^{14}C]Ac-CAT.

3.5. *Interaction between Ac-CAT and BA for binding to mitochondria*

BA interferes with the binding of AT or CAT to mitochondria [1]. Most likely, this is an indirect effect since BA and CAT (or AT) bind to distinct

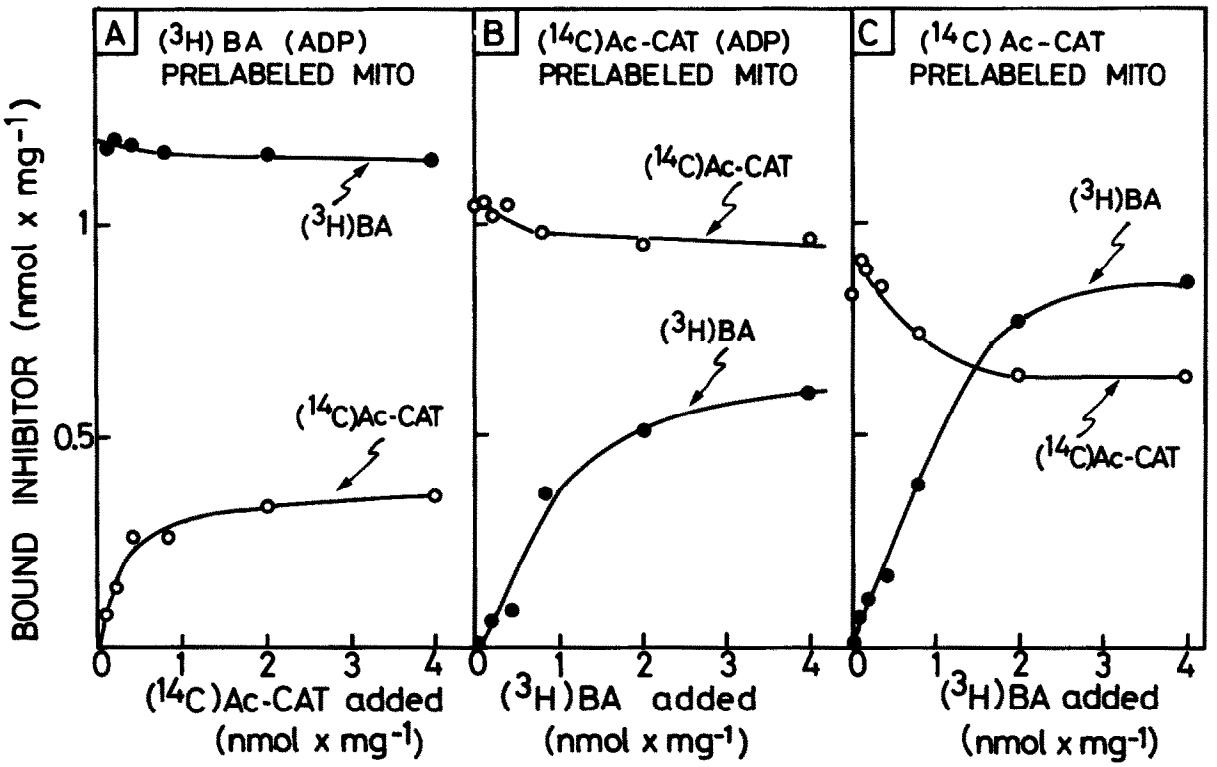

Fig.7. Interaction between [^{14}C]Ac-CAT and [^{3}H]BA for binding to the ADP/ATP carrier. (A) Rat heart mitochondria were labeled by preincubation with [^{3}H]BA (2 nmol/mg protein) in the presence of 10 μM ADP for 30 min at 0°C. Then [^{14}C]Ac-CAT was added at increasing concentrations and incubated for another 30 min at 0°C. (B) Rat heart mitochondria were preincubated with [^{14}C]Ac-CAT (2 nmol/mg protein) in the presence of 10 μM ADP at 0°C for 30 min. Then [^{14}C]BA was added at increasing concentrations and incubated for another 30 min at 0°C. (C) Same conditions as in (B), except that ADP was added with [^{3}H]BA (displacement step) instead of with [^{14}C]Ac-CAT (prelabeling step). In all cases, incubations were terminated by centrifugation, and the radioactivity of the pellets was measured.

sites on the ADP/ATP carrier. The stoichiometry of the interaction has not yet been determined. To solve this problem, a double labeling experiment based on the use of [^{3}H]BA and [^{14}C]Ac-CAT was carried out. In the first assay (fig.7A), heart mitochondria were preincubated with ADP with a concentration of [^{3}H]BA sufficient to saturate the high affinity sites (1.2 nmol/mg protein); then [^{14}C]Ac-CAT was added at increasing concentrations up to 4 nmol/mg protein. Under these conditions, the amount of bound [^{3}H]BA that was released was <0.1 nmol/mg protein, but a much greater amount of [^{14}C]Ac-CAT was found to bind with a saturation plateau corresponding to 0.35 nmol/mg protein. The total amount of bound [^{3}H]BA and [^{14}C]Ac-CAT was 1.5 nmol/mg protein, a value significantly in excess of the number of high affinity sites for either BA or Ac-CAT (1–1.2 nmol/mg protein). In the second assay (fig.7B) the heart mitochondria were prelabeled with a saturating concentration of [^{14}C]Ac-CAT (1.05 nmol bound/mg protein), and then [^{3}H]BA was added at increasing concentrations up to 4 nmol/mg protein. Here again, the amount of Ac-CAT released (0.1 nmol/mg protein) was much less than the amount of bound [^{3}H]BA at saturation (0.7 nmol/mg protein; and the total amount of bound ligands (Ac-CAT and BA) reached 1.6 nmol/mg protein. Other displacement experiments were done, differing from the above by addition of ADP during the displacement step. No significant difference was found for displacement of bound [^{3}H]BA by [^{14}C]Ac-CAT whether ADP was added with [^{3}H]BA or with [^{14}C]Ac-CAT (not shown). In contrast, a larger amount of bound [^{14}C]Ac-CAT was released following addition of [^{3}H]BA in the presence of ADP (fig.7C), than when ADP was included in the prelabeling medium together with [^{3}H]BA (fig.7B); even in this case, the amount of bound [^{3}H]BA exceeded the amount of [^{14}C]Ac-CAT released.

4. Discussion

This paper describes a convenient and rapid method to prepare a radioactive acetylated derivative of CAT of high specific radioactivity (Ac-CAT). This derivative mimics CAT by its binding and inhibitory properties. Chemically labeled Ac-CAT can therefore advantageously replace biosynthetically labeled CAT. This is the first report describing the synthesis of a CAT derivative with a high specific radioactivity, which can act biologically as a substitute of CAT.

As shown by mass spectroscopy, it is the primary alcohol group of the glucose moiety of CAT which is acetylated by acetic anhydride to give Ac-CAT. Esterification of the primary alcohol group does not alter the biological effects of CAT. This property was already found for AT and used to prepare fully active AT derivatives, including long chain acyl AT [16], succinyl AT [5], and azido-derivatives of AT [17]. Under the conditions described for preparation of Ac-CAT, only the primary alcohol group of the glucose disulfate moiety of CAT was acetylated, although its diterpene moiety contains an OH group. With higher amounts of acetic anhydride, diacetylated CAT accumulated; diacetylated CAT was much less active than Ac-CAT.

[^{14}C]Ac-CAT was used with [^{3}H]BA in double-labeling experiments to answer the question of whether a strict mutual exclusion occurs between CAT and BA for binding to the mitochondrial ADP/ATP carrier. Although some displacement of the bound ligand by the added antagonist ligand was observed, the total amount of bound [^{14}C]Ac-CAT and [^{3}H]BA exceeded the saturating level of binding for either of the two inhibitors, i.e., ~1 nmol/mg protein; this observation is consistent with the view that CAT and BA bind to different sites [18]. These sites are likely to interact with each other through propagated changes of conformation, so that the binding of Ac-CAT to its specific site leads to the release of the pre-bound BA and vice versa. However, mutual exclusion of one ligand by the other appeared to depend on experimental conditions and examples are provided where both CAT and BA sites are filled with their respective ligands. Another interesting observation concerns the effect of ADP on the displacement of bound Ac-CAT by BA. When ADP was added together with [^{14}C]Ac-CAT during the prelabeling step, the amount of [^{14}C]Ac-CAT released by addition of [^{3}H]BA (fig.7B) was much less than when ADP was added with [^{3}H]BA during the displacement step (fig.7C). This is probably related to the different specific conformations assumed by the ADP/ATP carrier in the presence of Ac-CAT and BA [19] and to the possibility that ADP renders the Ac-CAT and BA conformations more rigid.

Acknowledgements

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