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INTERACTION OF THE FLUORESCENT ANALOGUE PALMITOYL-(1,N^6)ETHENO-CoA WITH MITOCHONDRIAL MALATE DEHYDROGENASE AND PHOSPHOLIPID VESICLES

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SUMMARY

A fluorescent analogue, palmitoyl-εCoA was shown to have a fluorescence lifetime (19.5 nsec.), polarization and absorption and emission characteristics useful for studying interactions with enzymes and with model membranes. The fluorescence lifetime was found to be wavelength dependent. The analogue was a better inhibitor (50% inhibition at ∿ 0.2 μM) than palmitoyl-CoA (50% inhibition at 0.5 μM) when bound to mitochondrial malate dehydrogenase (L-malate: NAD^+ oxido reductase E.C.1.1.137). The fluorescence depolarization when bound to this enzyme was less than that observed for binding to bovine serum albumin suggesting some mobility of the chromophore while bound. The changes in polarization upon titration with phosphatidylcholine (egg) vesicles were consistent with a partition of palmitoyl-(1,N^6etheno)CoA between vesicles and malate dehydrogenase. Such partition may have physiological consequences.

Palmitoyl-(1,N^6)ethenoCoA (Fig. 1) is a fluorescent analogue for fatty acyl CoA in which the fluorescence reporter group is ethenoadenine. This analogue shares the spectral properties of the intensely fluorescent ethenoadenine derivatives introduce by Segrest, Leonard and Barrio (1).

This analogue was chosen to further characterize the interactions between fatty acyl-CoA and enzymes related to fatty acid metabolism. Oleoyl-(1,N^6)ethenoCoA has been found to interact less strongly with the enzyme citrate synthase than oleoyl-CoA even though the detergency of the analogue was comparable to oleoyl-CoA. This observation was used to argue for the physiological significance of fatty

Abbreviations: (1,N^6)etheno = ε; thus (1,N^6)ethenoCoA is εCoA.

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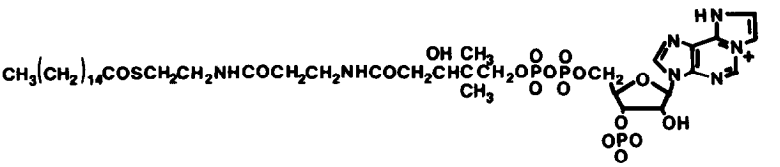


Fig. 1. Structure of Palmitoyl-(1,N^6)ethenoCoA.

acyl-CoA inhibition (2). Additional studies of the association of spin labeled fatty acyl CoA analogues with citrate synthase by one of us (3) have provided additional support for a specific, hence physiological association of these analogues with this enzyme.

Mitochondrial malate dehydrogenase [L-malate: NAD^+ oxidoreductase (E.C. 1.1.1.37)] was known to be even more sensitive to fatty acyl-CoA inhibition than citrate synthase (4,5). As will be shown, this enzyme is inhibited appropriately and at low concentrations of the fluorescent analogue palmitoyl-(1,N^6)etheno CoA. Fluorescence depolarization was then applied to study some features of the interaction of this fatty acyl-CoA with this enzyme.

MATERIALS AND METHODS

Palmitoyl-(1,N^6etheno)CoA was prepared from (1,N^6etheno)CoA (P-L Biochemicals, Inc.) and 99.9% palmitic acid (Sigma Chemical Co.) via the mixed carbonate anhydride 50, Sigma Chemical Co.) (7). The highly fluorescent band that cochromatographed with stearoyl-CoA (a quenching spot with Rf ~0.7) was scraped and eluted from the cellulose using 50/50 (v/v) isopropanol:water. This procedure was the same as employed for synthesizing spin labeled fatty acyl-CoA (3). The product was identified by the characteristic ultraviolet spectrum of the ε-adenine moiety (1). Some initial results were obtained using a highly purified mitochondrial malate dehydrogenase which was the gift of Dr. R. G. Wolfe, University of Oregon. The experiments reported here were carried out using highly purified, crystalline mitochondrial malate dehydrogenase which was the gift of Dr. L. J. Banaszak, Washington University School of Medicine, St. Louis. This enzyme also showed very low tryptophan fluorescence, a good criteria of purity (8). Protein was estimated from $\varepsilon^{280}_{1\%} = 2.7$ as suggested by those workers. The activity was estimated using the assay previously described (5).

Estimates of the nanosecond fluorescence life time were carried out using the Ortec 9200 1-ns fluorimeter. The excitation was carried out at 365 nm (50% transmission above 345 nm and below 380 nm) using a Corning 7-83 band-pass filter, or at 280 nm using an Optical Coating Laboratory, Inc. 51 N 6285 interference filter (50% transmission above 275.8 nm and below 292.6 nm). Emission was detected above 430 nm using the Corning 3-72 cut-off filter. The life time was estimated from graphs of intensity vs channel number (time) (9,10). Quinine sulfate whose lifetime was known (19.4 nsec) (11) was used to calibrate the apparatus.

Fluorescence polarization measurements were carried out in an apparatus similar to that described earlier (12). Illumination was provided by a Schoeffel Instruments Co. zenon lamp (200 watts) as a light source with the exciting light wavelength selected using Schoeffel Instruments quartz prism monochromator. The jacketed cell holder on an optical bench was modified to hold a 0.5 ml quartz cell. The fluorescence was detected at 90° through a Corning 3-373 cut-off filter passing light longer than 410 nm. The photomultiplier tube (EMI 9502B) signal was read from a digital voltimeter.

Fluorescence measurements were also made using a spectrofluorometer constructed from Bausch & Lomb grating monochromators and using a zenon lamp, photomultiplier tube and digital voltimeter as described earlier (12).

Mitochondrial phospholipid was prepared by extraction of heart particles (13), with $CHCl_3$ and CH_3OH (14). Vesicles were prepared from the solvent free lipid in 10 mM Tris HCl (pH 7.4) by probe sonication under an argon atmosphere (15).

RESULTS AND DISCUSSION

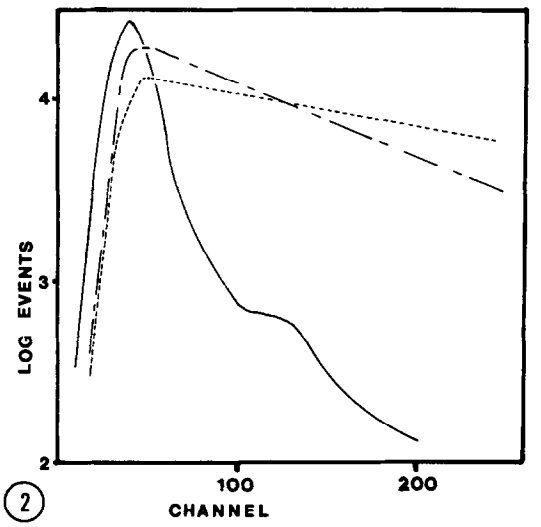
The fatty acyl-CoA analogue employed in these studies, palmitoyl-εCoA, was similar to the oleoyl-εCoA described earlier (2). The analogue was highly fluorescent showning absorption and fluorescence excitation near 275 nm but with maximum fluorescence excitation occuring at 311 nm. The maximum emission was broadly centered around 415 nm.

The nanosecond fluorescence lifetime for palmitoyl-εCoA (Fig. 2) when excited by light near 365 nm was estimated as 19.5 nsec, very close to the lifetime observed for quinine sulfate (19.4 nsec) under the same conditions. However when the excitation wavelength was decreased to 280 nm the observed lifetime (Fig. 2) for palmitoyl-εCoA increased to 43.3 nsec. The lifetimes for εATP observed under these same conditions were 27.1 nsec as previously observed (1,16) when excited at either 280 nm or at 365 nm. This wavelength dependence for the lifetime of palmitoyl-εCoA was unanticipated and may be related to the micellar properties of this analogue (2).

Polarization is defined as

$$P = \frac{I_{11} - I_{\perp}}{I_{11} + I_{\perp}}$$

where I_{11} and $I_{\perp}$ refer to the intensities of fluorescence measured with the analyzer parallel or perpendicular respectively to the plane of polarization of the incident excitation light. The polarization of 85 μm palmitoyl-εCoA excited



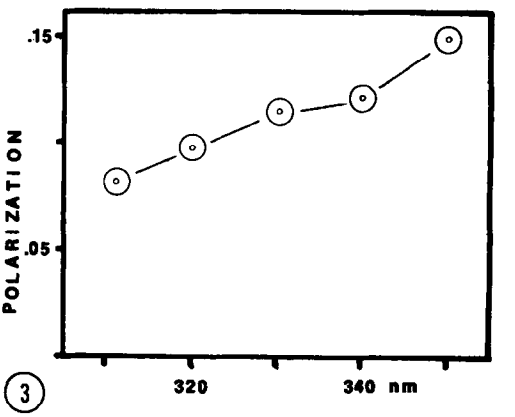
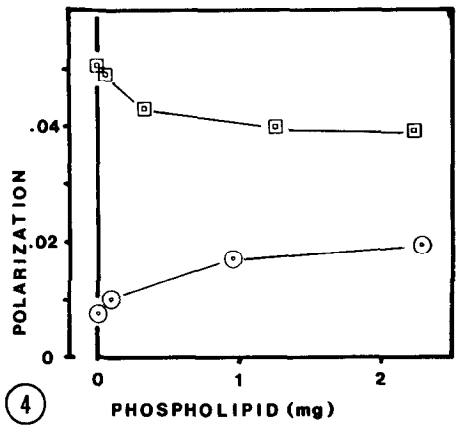


Fig. 2. Nanosecond Fluorescence Lifetime for Palmitoyl-εCoA. Palmitoyl-εCoA was present at 48.9 μM in 10 mM Tris·HCl (pH 7.4). This concentration exceeds the critical micelle concentration of 3-4 μm (2). The dotted line corresponds to excitation at 280 nm, the dashed line to excitation at 365 nm. The solid line was the lamp current. The emission filter was the same for all measurements.

Fig. 3. Wavelength Dependence of Polarization of Palmitoyl-εCoA Bound to Bovine Serum Albumin. The size of the small circles is the average deviation of five successive measurements of the polarization at the given wavelength. The palmitoyl-εCoA (85 μM) and the albumin (113 μM) were dissolved in 10 mM Tris·HCl (pH 7.4). No polarization was detectable over these wavelengths in the absence of palmitoyl-εCoA.

near 311 nm and measured beyond 410 nm was very low (P = 0.0077 ± 0.0006) as expected for a small molecule experiencing rapid intra- and intermolecular diffusion and partition between micellar and monomeric forms. Since no further attempt will be made to distinguish self aggregated palmitoylεCoA from monomeric forms all these forms will be referred to as "free".

Palmitoyl-εCoA, when bound to bovine serum albumin and excited at 311 nm showed a polarization of 0.0811 ± 0.005, an appreciable polarization compared with palmitoyl-εCoA free in solution, but smaller than anticipated from the polarization (0.27) of a chromophore like 8-anilino-1-naphtalensulfonic acid bound by bovine serum albumin (17). However when the wavelength dependence of polarization of palmitoyl-εCoA bound by bovine serum albumin was explored, the polari-



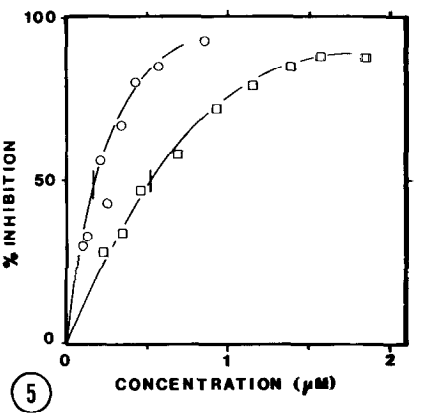


Fig. 4. Polarization of Palmitoyl- CoA in the Presence of Phospholipid Vesicles and Malate Dehydrogenase. Palmitoyl-εCoA (85 μM) in 10 mM Tris·HCl (pH 7.4) was titrated with the given amount of mitochondrial phospholipid vesicles (circles) in an initial volume of 0.53 ml. The experiment was repeated with 85 μM palmitoyl-εCoA together with 90 μM malate dehydrogenase (squares). The size of the small symbol is an estimate of the average deviation of five measurements of polarization at the given phospholipid concentration.

Fig. 5. Inhibition of Malate Dehydrogenase by Palmitoyl-εCoA and Palmitoyl-CoA. Palmitoyl-εCoA (circles) was a slightly better inhibitor than palmitoyl-CoA (squares). The assays were carried out as described previously (5) using 12 μg crystalline malate dehydrogense. The concentrations found to be required for 50% inhibition were 0.16 μM for palmitoyl-εCoA and 0.52 μM for palmitoyl-CoA.

zation was found to increase with longer wavelength (Fig. 3) much as has been previously observed for other etheno adenylates (1,16).

Increasing amounts of mitochondrial phospholipid vesicles increased the polarization of palmitoyl-εCoA (Fig. 4). The emission anisotropy, $\bar{A}$, will be the sum of the anisotropies (12) of free palmitoyl-εCoA and palmitoyl-εCoA associated with vesicles:

$$\bar{A} = \alpha.A_1 + (1 - \alpha) \quad A_2$$

where α is the fraction of the total anisotropy contributed by the palmitoyl-εCoA associated vesicles. The vesicle anisotropy is A_1 and A_2 is the anisotropy of palmitoyl-εCoA free in solution. The anisotropy is defined by:

$$A_i = 2P_i/3-P_i$$

where P_i is the polarization of the i^{th} species. If the maximum polarization of

palmitoyl-εCoA in the presence of the highest concentration of vesicles shown in Fig. 4 is taken as the polarization of palmitoyl-εCoA associated with vesicles, the fraction of palmitoyl-εCoA associated with vesicles at intermediate concentrations may be estimated from this value and that of free palmitoyl-εCoA. Thus in the presence of 950 μg phospholipid the fraction of anisotropy contributed by vesicle associated palmitoyl-εCoA is still 81% given the above assumptions.

Palmitoyl-εCoA was a good inhibitor for mitochondrial malate dehydrogenase (50% inhibition at ∿0.2 μM), even better than the natural analogue palmitoyl-CoA (50% inhibition at 0.5 μM) (Fig.5.) The polarization of palmitoyl-εCoA in the presence of malate dehydrogenase was higher (Fig. 4) than palmitoyl-εCoA free in solution but not as high as when bound by bovine serum albumin (Fig. 3). The lifetime for palmitoyl-εCoA was unchanged by the presence of malate dehydrogenase (not shown) and the concentrations of enzyme and palmitoyl-εCoA greatly exceeded that required for 50% inhibition. Thus the lower polarization for palmitoyl-εCoA bound to malate dehydrogenase compared with the value for bovine serum albumin suggests somewhat greater mobility of the fluorescent moiety while bound to the enzyme.

When phospholipid vesicles were added to palmitoyl-εCoA in the presence of malate dehydrogenase the polarization decreased. The magnitude of this decrease can be related to the relative affinity of palmitoyl-εCoA for malate dehydrogenase and phospholipid vesicles using the relations developed previously. The previous results (Fig. 4) together with independent measurements using spin labeled fatty acyl-CoA analogues (Caggiano and Powell, unpublished) suggest that only a small fraction of the palmitoyl-εCoA will be free in the presence of enzyme and vesicles. Thus the observed anisotropy may be meaningfully expressed in terms of only two components:

$$\alpha_v = \frac{\bar{A} - A_E}{A_v - A_E}$$

where α_v is the fraction either associated with vesicles or free. The respective anisotropies, A_v and A_E are derived from the polarizations given in Fig. 4 in the

absence of vesicles (A_E = 0.0343) and at the appropriate phospholipid concentration (Av). $\overline{A}$ was the anisotropy in the presence of enzyme and at a given phospholipid concentration. Thus at the highest lipid concentration, 4.3 mg/ml, A_v = 0.0129 and $\overline{A}$ = 0.0265. The fraction of the palmitoyl-εCoA associated with vesicles, α_v, was 0.35. The remainer was associated with enzyme. This estimate assumes a constant fluorescent lifetime at the wavelength used. Palmitoyl-εCoA has the same lifetime in the presence of malate dehydrogenase as free but the lifetime in the presence of phospholipid was not tested.

These observations suggest that fatty acyl-CoA may partition between biological membranes and soluble enzymes in a physiologically meaningful way. This suggestion is consistent with the activity measurements of Sumper et al (18, 19).

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