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A kinetic investigation of the acyl-CoA oxidase reaction with the use of a novel spectrophotometric assay

Inhibition by acetyl-CoA, CoA and FMN

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A direct-reading spectrophotometric assay for acyl-CoA oxidase activity is described. The assay is based on the strong absorption at 300 nm of deca-2-*trans*,4-*cis*-dienoyl-CoA, the product of oxidation of dec-4-*cis*-enoyl-CoA. By use of this assay, acetyl-CoA, CoA and FMN were found to be inhibitors of acyl-CoA oxidase, but with distinctly different kinetic characteristics.

INTRODUCTION

Animal and plant tissues contain peroxisomes, which can β-oxidize fatty acids [1,2]. The first enzyme in the peroxisomal β-oxidation sequence is acyl-CoA oxidase, which utilizes FAD and O_2 to oxidize fatty acyl-CoA esters. The reaction products are 2-*trans*-enoyl-CoA esters and H_2O_2. This enzyme is probably a rate-limiting step of peroxisomal β-oxidation [3,4], and a potential regulatory site.

Acyl-CoA oxidase activity has been measured polarographically [3] or as H_2O_2 production. Rates of H_2O_2 production may be recorded spectrophotometrically at 235 nm, but the molar absorption coefficient of H_2O_2 is low (58 $M^{-1} \cdot cm^{-1}$) [5]. The use of a wavelength in the far-u.v. range is also difficult, because of high background absorbance.

The sensitivity for H_2O_2 detection can be increased by using a coupled assay. With the use of peroxidases and suitable chromophores, spectrophotometric [6], fluorimetric [7,8] and luminometric assays [9] have been devised. However, methods based on measurement of H_2O_2 generation have the disadvantages that catalase present in the sample may cause errors, if catalase activity is not completely inhibited [10].

A direct-reading spectrophotometric assay, however, is in general simpler to use. Also, no coupling enzyme or reagents, which must be titrated to find optimal concentrations, are required.

To study the kinetic properties of acyl-CoA oxidase, we required a relative simple, sensitive and robust assay. Deca-2-*trans*,4-*cis*-dienoyl-CoA has a peak of absorption at 300 nm [11]. We have developed an assay for acyl-CoA oxidase, making use of this characteristic. Here we present the assay procedure, and some results obtained by using it.

MATERIALS AND METHODS

Materials

CoA, acetyl-CoA, FAD, FMN and Triton X-100 were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Dec-4-*cis*-en-1-ol was purchased from ICN Pharmaceuticals, K & K Laboratories Division, Plainview, NY, U.S.A.

Dec-4-*cis*-enoic acid was synthesized from dec-4-*cis*-en-1-ol by oxidation with CrO_3 [12]. Dec-4-*cis*-enoyl-CoA was synthesized, and dec-4-*cis*-enoyl-CoA concentrations were determined, as described by Osmundsen *et al.* [13].

Peroxisomal fractions were isolated from livers of male albino Wistar rats, given dietary clofibrate (0.5%, w/w) for 10–14 days, by using Percoll density gradients [14]. Protein was measured with Pierce BCA protein assay reagent, with freeze-dried bovine serum albumin as standard.

Spectrophotometric assay procedure

Dec-4-*cis*-enoyl-CoA is converted into deca-2-*trans*,4-*cis*-dienoyl-CoA by the acyl-CoA oxidase. Because of the absorption peak of deca-2-*trans*,4-*cis*-dienoyl-CoA at 300 nm [11], the reaction can be monitored by recording the increase in absorbance at 300 nm with increasing time of incubation. The molar absorption coefficient at 300 nm of deca-2-*trans*,4-*cis*-dienoyl-CoA is 19900 $M^{-1} \cdot cm^{-1}$ [11].

The assays were run at 25 °C in 20 mM-Tris/HCl buffer, pH 8.00, containing 20 μM-FAD, 0.005% Triton X-100 and typically 5–20 μM-dec-4-*cis*-enoyl-CoA. The reaction was started by adding peroxisomal protein, usually about 300 μg/ml of assay mixture.

Kinetic analysis of experimental data

$V_{max.}$ and K_m, and the inhibitor constants K_i, were calculated by using the PENZYME program [15,16] kindly supplied by Dr. J. Garfinkel, Moore School of Electrical Engineering, Philadelphia, PA, U.S.A.

The rate data were fitted to a conventional Michaelis–Menten rate equation (Fig. 1). All sets of data from any one inhibitor study (except those presented in Fig. 3) were fitted to a model describing the type of inhibition observed. Regression analysis was carried out in two steps: an initial analysis using a Simplex procedure, followed by a Fletcher–Powell analysis using the Simplex parameter estimates as starting values [15,16].

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RESULTS

Data shown in Fig. 1 represent rates of reaction obtained with various concentrations of dec-4-*cis*-enoyl-CoA. This substrate gave an apparent $V_{max.}$ of 182±17 nmol/s per g of protein (mean±S.D., $n = 3$) and an apparent K_m of 9.3±1.3 μM (mean±S.D., $n = 3$). With concentrations up to 112 μM no substrate inhibition or activation was observed. The initial rate of reaction was linear with respect to concentration of protein (results not shown).

Acetyl-CoA and CoA were found to behave as competitive inhibitors with respect to dec-4-*cis*-enoyl-CoA (Figs. 2 and 3*a*). Acetyl-CoA showed purely competitive inhibition with an apparent K_i of 300±25 μM.

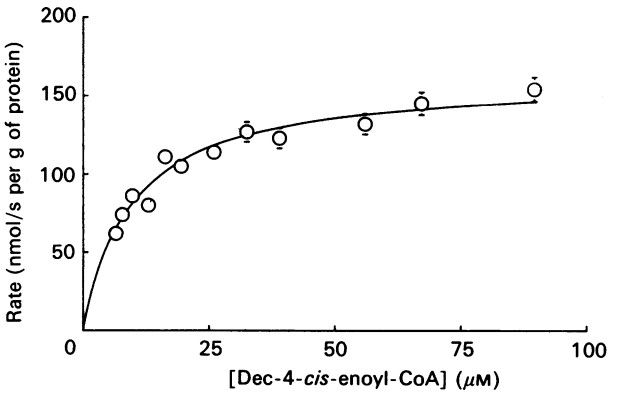

Fig. 1. Plot of rate of acyl-CoA oxidase activity versus dec-4-*cis*-enoyl-CoA concentration

Rates of dec-4-*cis*-enoyl-CoA oxidation by acyl-CoA oxidase were measured as described in the Materials and methods section. The curve shown represents the best fit suggested by the PENZYME program to the Michaelis–Menten equation. Error bars represent standard deviations ($n = 3$).

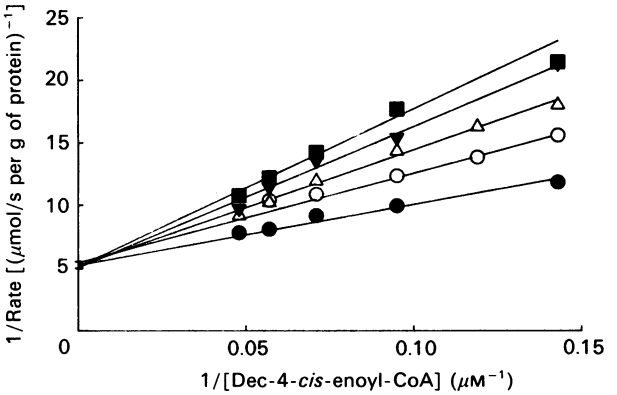

Fig. 2. Inhibition of acyl-CoA oxidase by acetyl-CoA

Double-reciprocal plot of rates of reaction versus various concentrations of dec-4-*cis*-enoyl-CoA. Acetyl-CoA was included in the assay mixtures to the concentrations indicated: ●, 0 μM; ○, 100 μM; △, 200 μM; ▼, 300 μM; ■, 400 μM. The lines represent the best fit of the data to a model where acetyl-CoA is a competitive inhibitor. The computed standard deviation in $V_{max.}$ is indicated as an error bar at the ordinate intercept. Experimental details are otherwise given in the Materials and methods section.

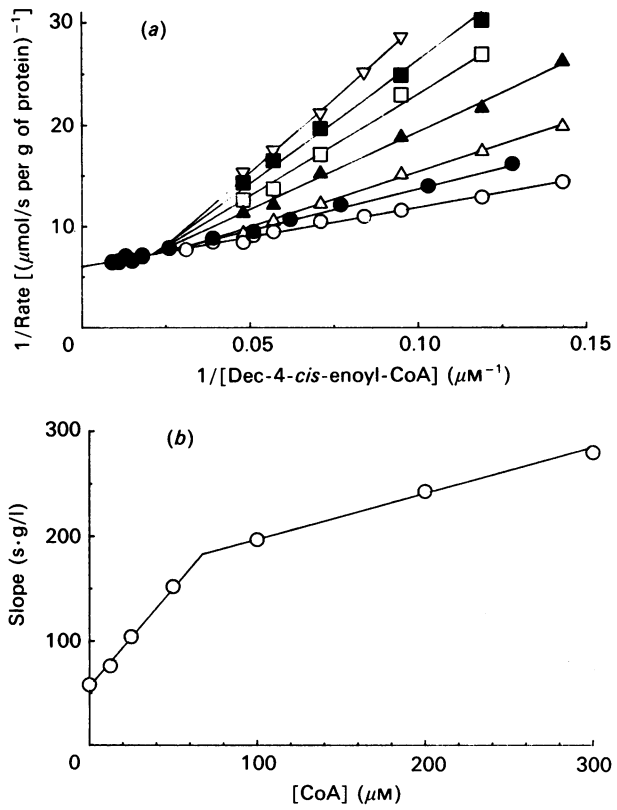

Fig. 3. Inhibition of acyl-CoA oxidase by CoA

(*a*) Double-reciprocal plot of rates of reaction versus various concentrations of dec-4-*cis*-enoyl-CoA. CoA was included in the assay mixtures to the concentrations indicated: ○, 0 μM; ●, 15 μM; △, 25 μM; ▲, 50 μM; □, 100 μM; ■, 200 μM; ▽, 300 μM. (*b*) Replot of slopes from (*a*) versus concentrations of CoA. Experimental details are otherwise given in the Materials and methods section. The lines drawn represents the best fits obtained by using linear regression on the $1/v$-against-$1/s$ data sets in (*a*), and on slope against [CoA] in (*b*).

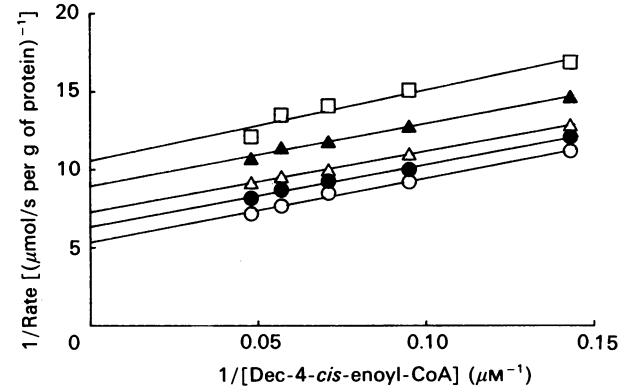

Fig. 4. Inhibition of acyl-CoA oxidase by FMN

Double-reciprocal plot of rates of reaction versus various concentrations of dec-4-*cis*-enoyl-CoA. FMN was included in the assay mixtures to the concentrations indicated: ○, 0 μM; ●, 100 μM; △, 200 μM; ▲, 400 μM; □, 600 μM. The lines represent the best fit to a model where FMN is an uncompetitive inhibitor. Experimental details are otherwise given in the Materials and methods section.

With CoA, in contrast, the inhibition was dependent on the concentration of CoA, as is evident from the biphasic replot of slope against [CoA] (Fig. 3*b*). At low concentrations of CoA one K_i of 30 ± 3 μM was obtained, and at higher concentrations of CoA another K_i of 320 ± 30 μM. In the Lineweaver–Burk plot the lines intersect to the right of the ordinate (Fig. 3*a*). At concentrations of substrate higher than this (50 μM) no inhibition or stimulation by CoA was observed.

FMN apparently behaved as an uncompetitive inhibitor of acyl-CoA oxidase, with dec-4-*cis*-enoyl-CoA as the substrate whose concentration was varied (Fig. 4), with an apparent K_i of 550 ± 7 μM.

DISCUSSION

Dec-4-*cis*-enoyl-CoA has a relatively high solubility in water compared with palmitoyl-CoA [17], the most frequent used substrate for acyl-CoA oxidase. Thus dec-4-*cis*-enoyl-CoA can therefore be used in assays without including bovine serum albumin, and without the problem of the substrate undergoing concentration-dependent phase transitions.

The K_m value of 9.3 μM for dec-4-*cis*-enoyl-CoA determined here is in the same range as the K_m values for decanoyl-CoA, lauroyl-CoA, myristoyl-CoA, palmitoyl-CoA, stearoyl-CoA, oleoyl-CoA and linoleoyl-CoA given by Osumi *et al.* [18]. A direct comparison may not be valid, as Osumi *et al.* [18] used the purified acyl-CoA oxidase, whereas we have used a peroxisomal fraction.

FMN is an apparent uncompetitive inhibitor with respect to acyl-CoA, with an apparent K_i of 550 μM. Uncompetitive inhibition is rare in biological systems [19]. Mixed type of inhibition may give an apparent uncompetitive pattern as a result of relatively low concentrations of FMN compared with the K_i for FMN, combined with the use of a relatively high concentration of FAD compared with the K_d of FAD of 0.6 μM [20].

Acetyl-CoA is the terminal product of β-oxidation, and behaves as a competitive inhibitor with respect to dec-4-*cis*-enoyl-CoA. The apparent K_i of 300 μM is probably too high for acetyl-CoA to have any regulatory role through feedback inhibition. Acetyl-CoA, being a substrate analogue, probably inhibits by binding to the acyl-CoA- (substrate-)binding site.

CoA also behaves as a competitive inhibitor of acyl-CoA oxidase. This inhibition appears to be biphasic with an apparent K_i of 30 μM at low CoA concentrations and another K_i of 320 μM at high CoA concentrations. This, taken together with the lack of inhibition at high substrate concentrations, suggests that CoA may have a regulatory function as regards the acyl-CoA oxidase. Physiologically, inhibition by CoA at low concentrations of substrate, and absence of inhibition at higher concentrations of substrate, would make sense.

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