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EFFECT OF AN EXOGENOUS SUCCINYL-CoA-GENERATING SYSTEM ON THE MEASUREMENT OF δ-AMINOLEVULINIC ACID SYNTHASE ACTIVITY IN RAT LIVER TISSUE BY A RADIOCHEMICAL ASSAY

STEVEN J. WOLFSON, ROSE M. ALLEN and JOSEPH R. BLOOMER *

Howard Hughes Medical Institute Laboratory, Yale University School of Medicine, 333 Cedar St., New Haven, CT 06510 (U.S.A.)

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Summary

Rat liver tissue was used to examine the effect of an exogenous succinyl-CoA-generating system on the radiochemical assay for δ-aminolevulinic acid synthase (succinyl-CoA:glycine *C*-succinyltransferase (decarboxylating), EC 2.3.1.37) activity developed by Ebert et al. (Ebert, P.S., Tschudy, D.P., Choudry, J.N. and Chirigos, M.A. (1970) Biochim. Biophys. Acta 208, 236—250). In the absence of exogenous succinate thiokinase, 34—62% (average 55%) of the radioactivity in the final column eluate could be attributed to δ-amino-[4-^{14}C]levulinic acid, as assessed by conversion of δ-aminolevulinic acid in the eluate to a pyrrole. The addition of succinate thiokinase markedly enhanced the formation of the contaminant(s), as succinyl-CoA was metabolized to a compound or compounds that eluted chromatographically with δ-aminolevulinic acid. This effect was abolished by 10 mM EDTA, probably because the generation of succinyl-CoA was suppressed due to the chelation of Mg^{2+}. These observations indicate that this radiochemical assay should be carefully examined for each set of assay conditions employed.

Introduction

δ-Aminolevulinic acid synthase (succinyl-CoA:glycine *C*-succinyltransferase (decaboxylating), EC 2.3.1.37) catalyzes the first, rate-limiting step in the bio-

* To whom correspondence should be addressed at the University of Minnesota School of Medicine, 420 Delaware St., S.E., Minneapolis, MN 55455, U.S.A.

Abbreviation: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid.

synthesis of heme, in which glycine condenses with succinyl-CoA [2—5]. Colorimetric methods [3—6] can be used to measure this enzymic activity when high levels are present. However, in order to measure the activity present in normal tissues sensitive radiochemical methods are required [1,7—11]. In some studies, maximal activity has been observed only when an exogenous system for generating succinyl-CoA has been used [9—11]. In the present study, the radiochemical assay for δ-aminolevulinic acid synthase activity developed by Ebert and coworkers [1] was examined when an exogenous succinyl-CoA-generating system was added to rat liver tissue.

Materials and Methods

Preparation of rat liver tissue. Male Sprague-Dawley rats (175—275 g), fed ad libitum, were killed by decapitation. The livers were perfused through the portal vein with ice-cold 0.25 M sucrose, 0.05 M Tris-HCl, 0.001 M EGTA, pH 7.4. A 10% (w : v) homogenate in sucrose/Tris/EGTA was prepared using a glass Potter-Elvehjem homogenizer and a motor-driven pestle run at 500 rev./min. The homogenate was centrifuged for 10 min at 700 × *g*, and the pellet was discarded. The supernatant was centrifuged at 6100 × *g* for 10 min, and the pellet was resuspended sequentially in sucrose/Tris/EGTA and sucrose/Tris, each time centrifugating at 6100 × *g*. The final suspension was in 0.25 M sucrose, 0.05 M Tris-HCl, pH 7.4. Electron microscopy of the fraction showed it to be enriched with structurally preserved mitochondria.

Studies were also done using the livers of male Sprague-Dawley rats which had been treated with allylisopropylacetamide. The animals were fasted for 20 h, and allylisopropylacetamide was administered intraperitoneally in a dose of 25 mg/100 g body weight. The animals were killed by decapitation 4 h later. Control animals received normal saline intraperitoneally.

Assay for δ-aminolevulinic acid synthase activity. δ-Aminolevulinic acid synthase activity was measured by the radiochemical assay of Ebert et al. [1], using 1—3 mg of protein from the mitochondrial fraction, or 10 mg wet weight from the whole homogenate. The assay mixture contained 1—2 μCi of [2,3-^{14}C]succinate (New England Nuclear, Boston, MA) in 50 μM succinate, 100 mM glycine, 0.2 mM pyridoxal phosphate, and 100 mM Tris-HCl, pH 7.4, in a final volume of 2 ml. Samples were run in duplicate. An enzyme blank, to which was added 0.5 ml of 25% trichloroacetic acid before the start of the incubation, was also run. Flasks containing 0.01 μCi of δ-amino[4-^{14}C]levulinic acid (New England Nuclear, Boston, MA) in 0.10 μM δ-aminolevulinic acid, with labeled succinate omitted, were included as a means of monitoring loss of δ-aminolevulinic acid during incubation and chromatographic isolation.

The reaction was terminated by adding 0.5 ml 25% trichloroacetic acid to the flask. The method of Ebert et al. [1] was used to isolate δ-aminolevulinic acid from the supernatant on columns of Dowex 50 (AG 50W-X8 resin from Biorad Laboratories, Richmond, CA) with the following modifications: (1) use of 4 : 1, rather than 2 : 1, methanol/0.1 M acetate buffer, pH 3.9, in a wash; (2) elution of δ-aminolevulinic acid with 4 ml of 0.1 M NaOH rather than 1 M NH_4OH. A portion of the NaOH eluate was counted in a Beckman LS-255 liquid scintillation counter with a counting efficiency of 73%, and the forma-

tion of δ-aminolevulinic acid was calculated after correcting for the loss of the δ-amino[4-^{14}C]levulinic acid standard (loss ranged from 13 to 25%).

The remaining portion of the NaOH eluate was combined with an equal volume of 1.0 M acetate buffer, pH 4.6. δ-Aminolevulinic acid in the sample was converted to a pyrrole and isolated on columns of Dowex 1 (AG 1-X8 resin from Biorad Laboratories, Richmond, CA) by the procedure of Marver et al. [12]. The NaOH eluate which contained the δ-amino[4-^{14}C]levulinic acid standard was processed identically in order to correct for degradation of δ-aminolevulinic acid which might have occurred during the elution with NaOH, as well as for the subsequent loss during conversion to a pyrrole. A second labeled standard was processed separately in order to determine the efficiency of the Marver procedure in the conversion and isolation of δ-aminolevulinic acid as a pyrrole (average 78%).

Source of succinate thiokinase activity. Succinate thiokinase was obtained from Porphyrin Products, Logan, UT. The enzyme has been partially purified from a mutant strain of *Rhodopseudomonas spheroides* which lacks δ-aminolevulinic acid synthase. Direct assay confirmed that the enzyme preparation did not contain δ-aminolevulinic acid synthase activity. Activity of the partially purified enzyme was measured using the succino-hydroxamic acid method of Kaufman et al. [13].

When succinate thiokinase was added to rat liver tissue during measurement of δ-aminolevulinic acid synthase activity, the reaction mixture contained 5 mM $MgCl_2$, 5 mM ATP, 5 mM glutathione, and 50 μM CoA in addition to the reagents used in the standard assay. An additional blank was assayed in which all constituents except rat liver tissue were included in the reaction mixture.

Results

The formation of δ-aminolevulinic acid in the mitochondrial fraction of rat liver was linear with the time of incubation through 25 min, and with protein through 3 mg. It was linear with whole homogenate through 10 mg wet weight. These conditions were employed for the assay. The addition of 10 mM EDTA to the reaction mixture increased the apparent formation of δ-aminolevulinic acid in the mitochondrial fraction from 547 ± 157 pmol/mg protein per h, mean ± S.D. of eight samples, to 737 ± 306, and in the whole homogenate from 398 ± 125 pmol/mg protein per h to 463 ± 178.

The addition of succinate thiokinase, in the absence of EDTA, greatly increased the apparent δ-aminolevulinic acid synthase activity in the mitochondrial fraction (Fig. 1). The activity measured in the presence of 0.2 unit of succinate thiokinase was more than 100 times that measured in its absence. Similar results were obtained with whole homogenate, as 0.2 unit of succinate thiokinase increased the apparent δ-aminolevulinic acid synthase activity to 32 nmol/mg protein per h.

However, when the final column eluate was examined further by converting δ-aminolevulinic acid to a pyrrole and isolating the pyrrole on a Dowex 1 column, it was found that the eluate was contaminated with other labeled compounds. In the absence of exogenous succinate thiokinase, 34—62% (average 55%) of the radioactivity was recovered in the pyrrole formed from

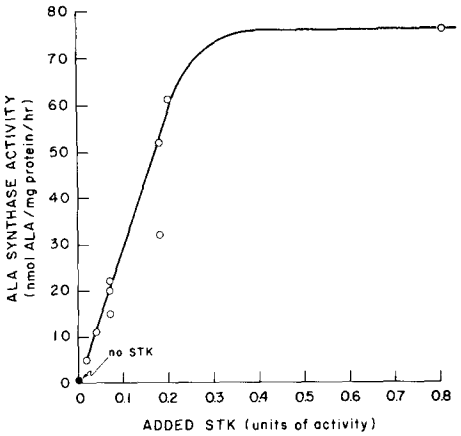

Fig. 1. Effect of a succinyl-CoA-generating system (STK) on apparent δ-aminolevulinic acid (ALA) synthase activity in rat liver mitochondria, measured by the radiochemical assay of Ebert et al. [1]. One unit of succinate thiokinase is that amount which will catalyze the synthesis of 1 μmol succinyl-CoA in 30 min.

δ-aminolevulinic acid. The large increase in the apparent activity of δ-aminolevulinic acid synthase observed upon addition of succinate thiokinase was due mainly to the increased formation of one or more of the labeled contaminants (see Table I for a representative study). Omission of glycine from the reaction mixture did not affect the synthesis of the species responsible for the contamination (Table I). However, CoA appeared to be crucial (Table I), as was the concentration of succinate (Fig. 2) and the presence of rat liver tissue. The contamination could not be attributed to [^{14}C]succinyl-CoA because labeled succinyl-CoA was not eluted with δ-aminolevulinic acid.

Addition of 10 mM EDTA abolished the formation of the labeled contaminant(s) (Fig. 3). The sharp drop in activity between 1 mM and 10 mM EDTA suggested that this was due to inactivation of succinate thiokinase by the chelation of Mg^{2+} to EDTA. However, this explanation may not be sufficient, since addition of excess Mg^{2+} restored succinate thiokinase activity (Fig. 4), but not the formation of the labeled contaminant(s).

TABLE I

EFFECT OF AN EXOGENOUS SUCCINYL-CoA-GENERATING SYSTEM ON δ-AMINOLEVULINIC SYNTHASE ACTIVITY IN RAT LIVER MITOCHONDRIA

	δ-Aminovulinic acid synthase activity (pmol/mg protein per h)	
	By method of Ebert et al. [1]	After adding method of Marver et al. [12]
No succinate thiokinase system	465	290
Succinate thiokinase added (0.2 unit)	61 400	920
Succinate thiokinase/glycine omitted	62 700	—
Succinate thiokinase/CoA omitted	1 765	180

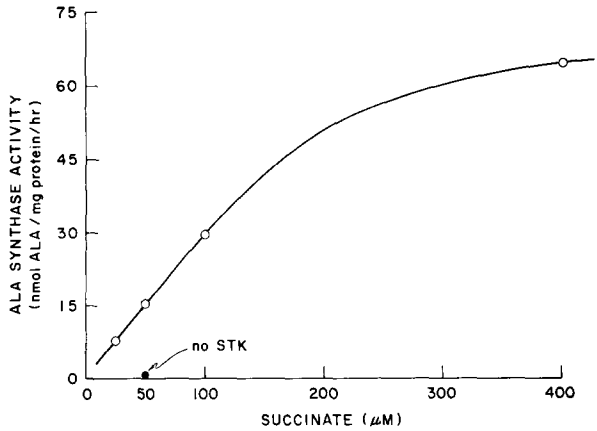

Fig. 2. Effect of the concentration of succinate on apparent δ-aminolevulinic acid (ALA) synthase activity in rat liver mitochondria, measured by the radiochemical assay of Ebert et al. [1]. Succinate thiokinase (STK) was added at 0.07 unit of activity.

In animals treated with allylisopropylacetamide, δ-aminolevulinic acid synthase activity in the mitochondrial fraction increased to 2640 pmol/mg protein per h, as measured without an exogenous source of succinate thiokinase. Under this condition, all of the label in the NaOH eluate was recovered as δ-aminolevulinic acid pyrrole.

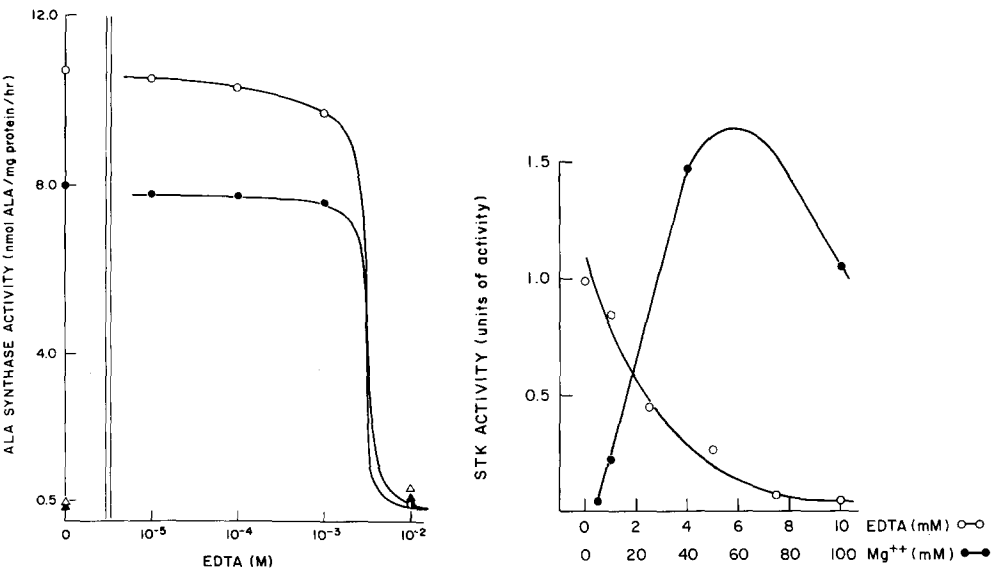

Fig. 3. Effect of EDTA on apparent δ-aminolevulinic acid (ALA) synthase activity in homogenate (●) and mitochondria (○), with (●, ○) and without (△, ▲) succinate thiokinase present. δ-Aminolevulinic acid synthase activity was measured by the radiochemical assay of Ebert et al. [1].

Fig. 4. Effect of EDTA on succinate thiokinase (STK) activity in the presence of 5 mM Mg^{2+} (○), and the overriding effect of adding excess $MgCl_2$ to reaction mixtures containing 10 mM EDTA (●).

Discussion

Accurate measurements are needed for study of the important regulatory enzyme δ-aminolevulinic acid synthase. The present results indicate that under certain conditions the radiochemical assay developed by Ebert et al. [1] may not provide adequate separation of δ-amino[^{14}C]levulinic acid from other labeled metabolites on Dowex 50 columns, causing an overestimation of enzyme activity.

In the absence of an exogenous source of succinyl-CoA generation, only 55% of the radioactivity in the final column eluate could be attributed to δ-amino-[4-^{14}C]levulinic acid, when δ-aminolevulinic acid was converted to a pyrrole and isolated on a Dowex 1 column. Although other investigators who used this radiochemical assay found that a significantly greater fraction of the radioactivity was in δ-aminolevulinic acid [1,10], a recent study using an amino acid analyzer also demonstrated that the final column eluate may contain labeled contaminants [14].

The degree and type of contamination may depend in part on the type of tissue examined [14]. In the present study, basal δ-aminlevulinic acid synthase activity was measured in hepatic tissue obtained from fed rats. When the enzyme activity was induced in the animals by fasting and administration of allylisopropylacetamide, the level of contamination was insignificant.

The use of an exogenous system to generate succinyl-CoA significantly increased the formation of one or more labeled contaminants, and only a small fraction of the label in the final column eluate could be attributed to δ-amino-levulinic acid when it was further characterized by conversion to a pyrrole. The contaminant was not identified, but it was apparently caused by the metabolism of succinyl-CoA to a compound or compounds that chromatographed similarly to δ-aminolevulinic acid. Addition of 10 mM EDTA decreased the amount of contamination, probably by chelating Mg^{2+} and thereby inactivating the succinate thiokinase. This interpretation is complicated by the fact that addition of excess Mg^{2+} restored succinate thiokinase activity without restoring generation of the contaminating species.

Assays of δ-aminolevulinic synthase activity generally utilize EDTA because it inhibits δ-aminolevulinic acid dehydrase activity and thereby prevents catabolism of product [5,15]. Previous studies demonstrated that EDTA may also enhance the formation of δ-aminolevulinic acid, possibly because mitochondria are stabilized [2,5,16]. If an exogenous succinyl-CoA-generating system is used in the assay, however, the effect of EDTA on succinyl-CoA generation must be examined separately under the conditions of the assay, since EDTA inhibits this Mg^{2+}-requiring enzyme

In light of the present studies, it is important to characterize the final product carefully for each set of assay conditions used to measure δ-amino-levulinic acid synthase activity by a radiochemical assay.

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