Determination of the quantity of acetyl CoA carboxylase by [^{14}C]methyl avidin binding

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Summary Conditions are described under which monomeric [^{14}C]methyl avidin binds to SDS-denatured biotin enzymes and remains bound through polyacrylamide gel electrophoresis. The location of radioactive proteins on the dried gel was determined by fluorography and their identity was established by subunit molecular weight. The relative quantity of bound radioactive avidin, stoichiometrically equivalent to the molar quantity of biotin protein, can be determined by scanning the fluorograph with a soft laser densitometer. To determine the absolute quantity of biotin protein, the radioactive areas of the dried gel were cut out, resolubilized, and assayed for radioactivity. Since the specific radioactivity of the [^{14}C]methyl avidin was known, the quantity of avidin bound and therefore the quantity of biotin enzyme could be calculated. The method is illustrated by the analysis of purified acetyl CoA carboxylase and is applied to the analysis of biotin enzymes in isolated rat liver mitochondria.—**Roman-Lopez, C. R., J. Goodson, and J. B. Allred.** Determination of the quantity of acetyl CoA carboxylase by [^{14}C]methyl avidin binding. *J. Lipid Res.* 1987. **28:** 599–604.

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Acetyl CoA carboxylase, like most other enzymes, is normally detected in tissue preparations by measurement of activity. However, enzyme activity often cannot be equated with quantity, especially with acetyl CoA carboxylase since the activity of this enzyme is markedly affected by a number of factors, including citrate (1), malonyl CoA (1), monovalent ions (2), covalent modification (3, 4), and proteolysis (5). Furthermore, although acetyl CoA carboxylase has long been thought to be located exclusively in cytoplasm (6), recent evidence indicates that there are particulate forms of the enzyme (7, 8) including a relatively inactive mitochondrial form (9). Thus, understanding of the regulation of this enzyme requires a method for measurement of enzyme quantity as well as activity.

Immunotitration has been the only method available to estimate the quantity of acetyl CoA carboxylase, but this method is, at best, semi-quantitative and in the final analysis must rely upon measurement of activity of the enzyme either precipitated or not precipitated by antibody (8, 10). The discovery that [^{14}C]methyl avidin would bind to SDS-denatured acetyl CoA carboxylase and remain bound through PAGE (11) provided an opportunity for the development of a method for a much more accurate determination of the quantity of enzyme, based upon biotin content rather than enzyme activity. The validity of the method is established here by the analysis of known quantities of purified acetyl CoA carboxylase. The method was then applied to measurement of the quantity of biotin enzymes in mitochondrial preparations.

MATERIALS AND METHODS

Enzyme preparations

Acetyl CoA carboxylase was purified from rat liver by polyethylene glycol precipitation and chromatography on Sepharose 4B (4) and stored at −20°C. The enzyme was phosphorylated by incubation with [γ-^{32}P]ATP (200 cpm per pmol) in the presence of cyclic-AMP-dependent protein kinase catalytic subunit (4). Mitochondria were prepared from liver of fasted rats (48 hr) (9) by differential centrifugation (12).

Protein denaturation

The standard procedure for SDS denaturation of proteins was to add the enzyme preparation or tissue extract to two volumes of hot (95°C) SDS reaction mixture to give a final concentration of 3.3% SDS, 1.3% 2-mercaptoethanol, and 10% sucrose, along with a small amount of bromophenol blue indicator. Heating was continued for at least 4 min. The purified acetyl CoA carboxylase had bovine serum albumin added (40 mg per ml) before SDS denaturation. Mitochondria were solubilized by the addition of Triton X-100 (2% final concentration) immediately before SDS denaturation. Protein concentration in the purified enzyme and solubilized mitochondrial preparations was measured by dye binding (13) using bovine serum albumin as a standard.

Protein in frozen–thawed preparations of purified acetyl CoA carboxylase tends to coagulate which can make sampling difficult. In one experiment (see Fig. 4) an alternative procedure was used in which SDS (2% final concentration) was added to a suspension of the enzyme and the mixture was heated (95°C) for 5 min. After appropriate dilution of an aliquot with water, protein was determined by a modification of the Lowry method (14), using similarly treated bovine serum albumin as a standard. The SDS-solubilized enzyme had bovine serum albumin and SDS-reaction mixture added to provide the concentrations of reactants given above and was reheated (95°C) for 4 min.

Avidin incubation

SDS-denatured enzyme, potassium ion (as potassium acetate) stoichiometrically equivalent to the quantity of SDS, avidin or [^{14}C]methyl avidin, and 50 μM sodium phosphate buffer (pH 7.0) were combined to give a final

Abbreviations: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

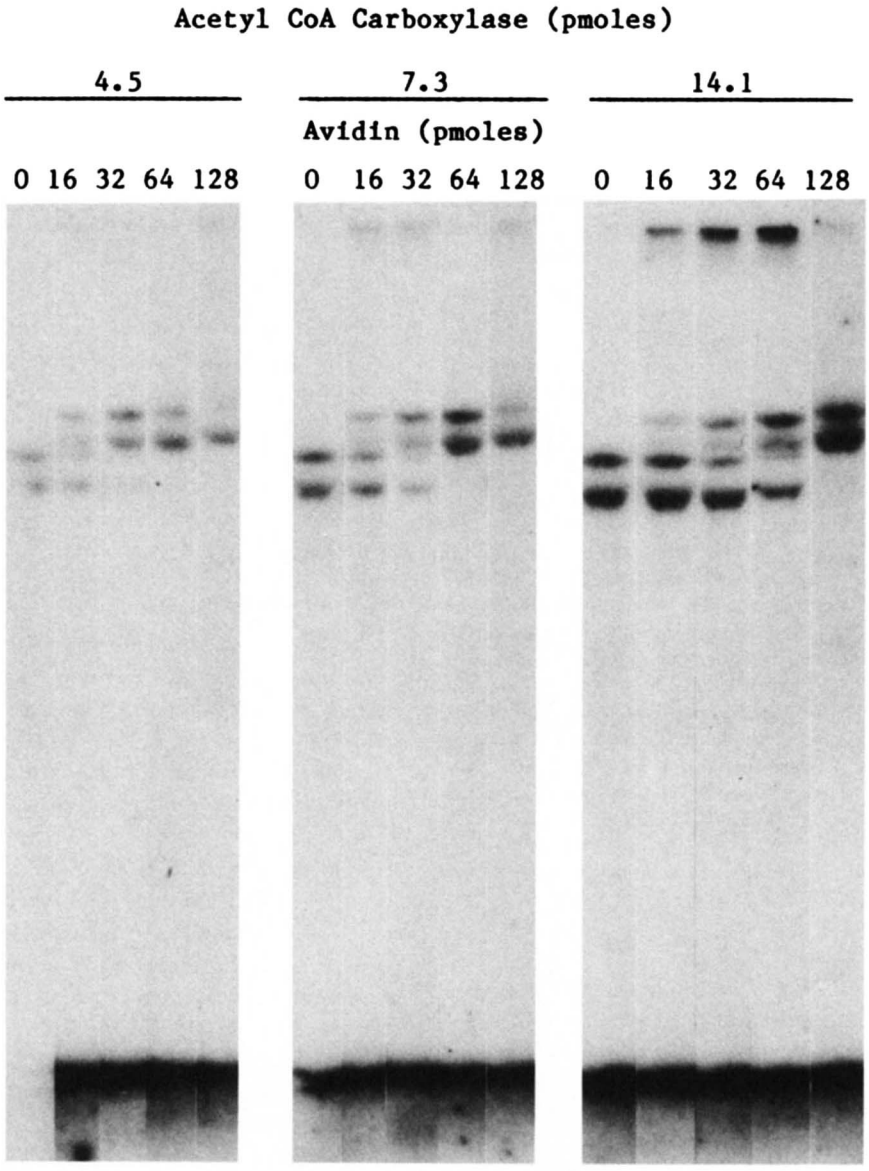

Fig. 1. Autoradiograph of SDS-denatured ^{32}P-labeled purified acetyl CoA carboxylase preincubated with increasing amounts of avidin before SDS-PAGE. The quantities shown are those applied to each channel of the gel.

volume of 50 μl. After incubation for 4 hr at 37°C, 20 μl of 20% sucrose in 0.15 M Tris-chloride buffer, pH 6.8, was added and the mixture was stored at 0–4°C overnight.

PAGE and fluorography

A 5% polyacrylamide slab gel with a 3% polyacrylamide stacking gel was prepared (15) and 50 μl of the avidin incubation mixture was placed in each channel. Current (7 mA) was applied overnight. After electrophoresis, the gel was stained with Coomassie Blue (15) and destained with methanol–acetic acid–water 50:10:40. For autoradiography of the ^{32}P-labeled enzyme, the gel was dried on filter paper with heat (60°C) and vacuum, using a slab gel drier. The dried gel was exposed to X-ray film with an intensifying screen before the film was developed according to the suggestions of the manufacturer. For fluorography of [^{14}C]-methyl avidin, the destained gel was submerged in scintillation solution before it was dried. The dried gel was exposed to X-ray film at −20°C before the film was developed.

Determination of quantity of avidin remaining bound through SDS-PAGE

Relative amounts of radioactivity in protein bands were determined by scanning the autoradiograph or fluorograph with a soft laser densitometer connected to a computer. For a more direct determination of [^{14}C]methyl avidin bound

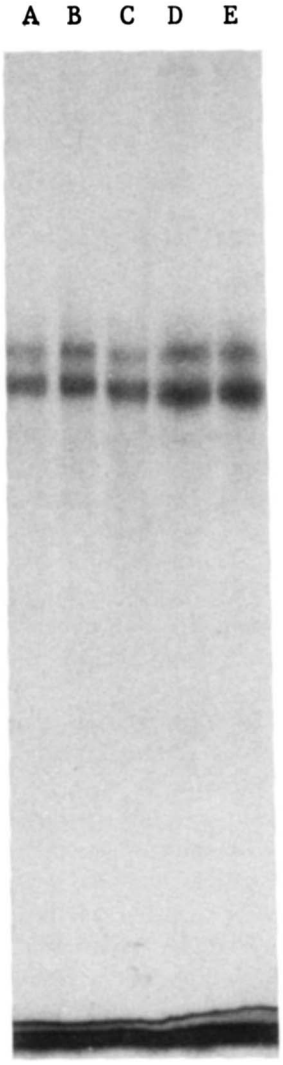

Fig. 2. Fluorograph of increasing amounts of SDS-denatured purified acetyl CoA carboxylase preincubated with [^{14}C]methyl avidin (45,000 cpm, 200 cpm/pmol) before SDS-PAGE. One of three replications is shown. The quantity of enzyme protein (μg) added to each gel channel was: A, 0.47; B, 0.71; C, 0.94; D, 1.41; E, 2.12.

to biotin proteins, a fluorograph was used to locate the radioactive areas on the dried gel. These areas were cut out and incubated in 30% hydrogen peroxide, 5% ammonium hydroxide at 50°C to resolubilize the acrylamide and protein (16). Incubation was continued (about 48 hr) until most of the hydrogen peroxide had decomposed. Scintillation solution (9) was added and radioactivity was determined before and after the addition of an internal standard to correct for quenching.

Materials

Scintillation solution for fluorography (Enlighten), [γ-^{32}P]ATP and [^{14}C]methyl avidin (New England Nuclear, Boston), SDS, electrophoresis chemicals, and slab gel drier (Bio-Rad, Richmond, CA), avidin, and other biochemicals (Sigma Chemical Company, St. Louis), and X-ray film (Eastman-Kodak, Rochester) were obtained from the indicated sources. The soft laser densitometer was purchased from Biomed Instruments, Fullerton, CA.

RESULTS AND DISCUSSION

Acetyl CoA carboxylase purified from rat liver by polyethylene glycol precipitation has been shown by SDS-PAGE to contain two different subunit forms of the enzyme whose molecular weights differ by only a few thousand (4). Both of the subunit forms incorporated covalently bound radioactive phosphate when the enzyme was incubated with [γ-^{32}P]ATP in the presence of purified cyclic-AMP-dependent protein kinase catalytic subunit (4). Autoradiography (**Fig. 1**) shows that when such a ^{32}P-labeled acetyl CoA carboxylase preparation was preincubated with avidin before SDS-PAGE the subunit molecular weight of both forms of the enzyme increased. We have previously established (11) that the increase in subunit molecular weight brought about by avidin incubation was approximately 16,000, the molecular weight of monomeric avidin (17). It is important to note that it is the monomeric form of avidin and not the native tetrameric form that remains bound to the biotin of subunits of acetyl CoA carboxylase because the two forms have very different binding constants for biotin. While tetrameric avidin has a very high affinity for biotin, the complex formed between monomeric avidin and biotin is

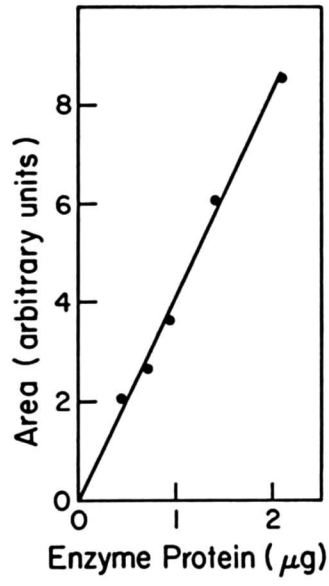

Fig. 3. Relative density of the fluorograph shown in Fig. 2 as measured by soft laser densitometry. Each point is the average of three replications.

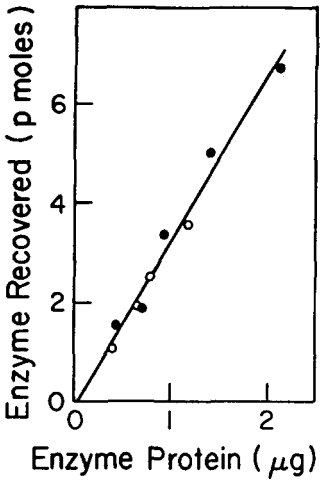

Fig. 4. The quantity of SDS-denatured purified acetyl CoA carboxylase recovered from dried gel as measured by [^{14}C]methyl avidin remaining bound through SDS-PAGE. In experiment 1 (closed circles), the gel was the same as that used to produce the fluorograph in Fig. 2. In experiment 2 (open circles), protein was determined after SDS-denaturation as described in text. Each point is an average of three replications in experiment 1 and four replications in experiment 2.

much less stable (18). In spite of this lower stability, Fig. 1 shows that the complex was sufficiently stable to survive SDS-PAGE.

It is also clear from Fig. 1 that the quantity of avidin required in the preincubation mixture to force the formation of the avidin-biotin enzyme complex to completion increased as the quantity of acetyl CoA carboxylase increased. The apparent dissociation constant of the reaction, calculated from the relative amounts of unreacted enzyme and the complex as measured by soft laser densitometry, was about 0.3 μM. This value is in reasonably close agreement with previous estimates of the stability of monomeric avidin-biotin complex, measured when monomeric avidin was coupled to Sepharose. Green (18) estimated the dissociation constant to be about 0.1 μM, and Kohanski and Lane (19) gave an estimate of approximately 0.2 μM. When avidin was not in excess, particularly with relatively large amounts of acetyl CoA carboxylase as in Fig. 1, up to four biotin enzyme subunits may bind to the same tetrameric avidin molecule, resulting in a protein too large to enter the separating gel. It has been shown that tetrameric avidin saturated or partially saturated with biotin resists dissociation into subunits (18).

The fact that under appropriate conditions monomeric avidin binds to the biotin prosthetic group of SDS-denatured proteins and remains bound through SDS-PAGE provided an opportunity to develop a method for the quantitative determination of biotin enzymes. **Fig. 2** shows a fluorograph in which increasing amounts of SDS-denatured, purified rat liver acetyl CoA carboxylase were preincubated with an excess of [^{14}C]methyl avidin before SDS-PAGE. Both protein bands bound radioactive avidin in agreement with our previous report (11). The density of the bands on X-ray film increased as the quantity of acetyl CoA carboxylase in the preincubation mixture increased (**Fig. 3**) as determined by scanning the X-ray film with a soft laser densitometer coupled to a computer that calculated the area under the curves. The summation of the area of the scan of the two peaks showed a linear relationship (r = 0.99) with the quantity of acetyl CoA carboxylase in the preincubation mixture.

The densitometry data provide an estimate of the relative amount of biotin enzyme. A second method (**Fig. 4**) was used to assess the quantity of radioactive avidin bound to biotin enzymes which provides an estimate of the absolute quantity of these enzymes. The exposed X-ray film (Fig. 2) was used to locate the radioactive areas on the dried gel which were cut out, resolubilized, and assayed for radioactivity. The molar quantity of radioactive avidin bound was then calculated, based upon the known specific activity of the [^{14}C]methyl avidin. Since it is known that acetyl CoA carboxylase contains one mole of biotin per mole of enzyme subunit (17) and that one mole of monomeric avidin binds one mole of biotin (18), the molar quantity of bound radioactive avidin is exactly equal to the molar quantity of acetyl CoA carboxylase.

Fig. 4 also shows the result of a second experiment in which protein was determined after the enzyme preparation was boiled in SDS. In both experiments, there was a high correlation between the molar quantity of enzyme recovered and protein added (r = 0.98). Since this analysis was performed using purified acetyl CoA carboxylase, Fig. 4 represents a standard curve. Assuming an average subunit molecular weight of 250,000 for the acetyl CoA carboxylase (11), the recovery was 85%.

The fact that avidin bound to the biotin of subunits of purified acetyl CoA carboxylase after SDS-denaturation suggests that the method is equally applicable for the determination of the quantity of this enzyme in SDS-denatured crude preparations. The method should also be applicable to the determination of the quantity of other biotin enzymes, because SDS-denaturation results in the unfolding of protein chains (20) which should make the biotin site accessible to the avidin. To establish that the method is applicable to crude preparations, proteins from isolated mitochondria were subjected to SDS-denaturation and biotin enzymes were labeled with [^{14}C]methyl avidin before PAGE. **Fig. 5**, left panel, shows that the quantity of radioactive avidin remaining bound to biotin protein subunits through electrophoresis was directly proportional to the quantity of mitochondrial protein preincubated with [^{14}C]methyl avidin. A fluorograph of one of the replicates used for collection of the data shown in the left panel is shown in Fig. 5, right panel. The upper bands (A and B) have been identified as two different forms of acetyl CoA carboxylase on the basis of subunit molecular weight (9)

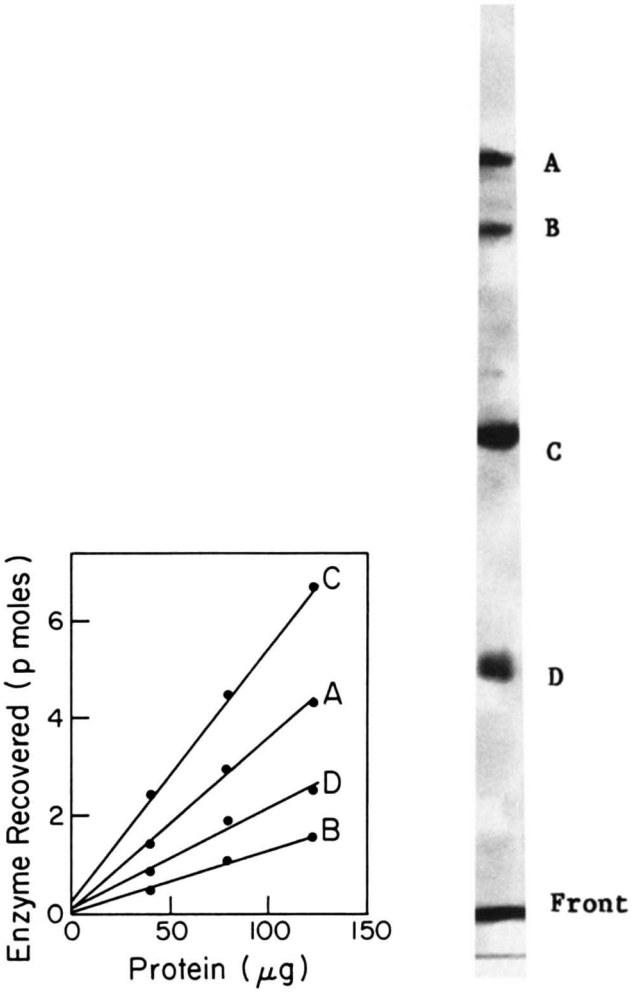

Fig. 5. Left: The quantity of mitochondrial biotin enzymes recovered as a function of mitochondrial protein preincubated with [^{14}C]methyl avidin. Each point is an average of three replications. Right: Fluorograph of one replicate at one level used for the analysis in left panel. The identity of the enzymes are: A and B, two forms of acetyl CoA carboxylase; C, pyruvate carboxylase; D, propionyl CoA carboxylase and 3-methyl crotonyl CoA carboxylase.

and the fact that both protein subunits are precipitated by antibody prepared from serum of sheep after injection of purified rat liver acetyl CoA carboxylase (J. B. Allred, unpublished work). Band C has been identified as pyruvate carboxylase (9) on the basis of molecular weight (21), while band D includes both propionyl CoA carboxylase and 3-methylcrotonyl CoA carboxylase (9, 21). Estimates of the quantity of each of the biotin proteins (pmol per mg of mitochondrial protein) as determined from the data in Fig. 5 are: acetyl CoA carboxylase, 34 and 13 for bands A and B, respectively; pyruvate carboxylase, 52; and the combination of propionyl CoA carboxylase and 3-methylcrotonyl CoA carboxylase, 20.

The accuracy of the method described here for the determination of absolute amounts of biotin enzymes depends upon the validity of several assumptions, not the least of which is that the specific activity of the [^{14}C]methyl avidin is known with certainty. It must also be assumed that all of the biotin enzyme becomes bound and remains bound to avidin through SDS-PAGE, the validity of which depends upon the dissociation constant for the avidin–biotin enzyme complex. For example, with the concentration of reactants used in the experiments shown in Fig. 4, if the dissociation constant was as low as 0.1 μM, less than 3% of the acetyl CoA carboxylase would have been unreacted and undetected. If, however, the dissociation constant was as high as 0.8 μM, 15% of the enzyme would have remained unbound. The apparent dissociation constant for experiment 1, Fig. 4 was 0.5 μM and that for experiment 2 was slightly higher. The lack of complete reaction likely accounts for the fact that recovery was only 85% even though previous results indicated that the purification procedure used produces a nearly homogeneous enzyme preparation (4).

Experience with this method has indicated that subtle changes in assay conditions can alter the apparent dissociation constant and therefore the extent to which the reaction goes to completion. Even so, evidence presented here indicates that the method provides a reasonably accurate measurement of the absolute quantity of acetyl CoA carboxylase in purified preparations. The method also provides an estimate of the absolute amount of this enzyme in mitochondria since it is highly unlikely that the presence of other SDS-denatured proteins would interfere with binding of avidin to the biotin in subunits of acetyl CoA carboxylase. Although the accuracy of the method for the determination of biotin enzymes in addition to acetyl CoA carboxylase has not been established because there are no independent methods to measure their quantity in crude tissue preparations, it is reasonable to expect that the method also provides an estimate of the absolute amounts of these enzymes. Even when this expectation is not realized, the linear increase in the quantity of radioactivity bound after SDS-PAGE with increasing amounts of mitochondrial protein (Fig. 5) indicates that the method can be used to compare the relative amounts of each of the biotin enzymes in tissue extracts as long as the analyses are done under identical conditions.

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