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LIPOIC ACID CONTENT OF DIHYDROLIPOYL TRANSACYLASES DETERMINED BY ISOTOPE DILUTION ANALYSIS

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SUMMARY. The content of protein-bound lipoyl moieties in the dihydrolipoyl transacylase component of pyruvate and α-ketoglutarate dehydrogenase complexes has been determined by isotope dilution analysis. The results show that the Escherichia coli dihydrolipoyl transsuccinylase and the bovine kidney and heart dihydrolipoyl transacetylase each contain one lipoyl moiety per polypeptide chain, whereas the E. coli dihydrolipoyl transacetylase contains two lipoyl moieties per chain.

The pyruvate and α-ketoglutarate dehydrogenase complexes are organized about a core, consisting of dihydrolipoyl transacetylase or dihydrolipoyl transsuccinylase, to which pyruvate dehydrogenase or α-ketoglutarate dehydrogenase and dihydrolipoyl dehydrogenase are joined by noncovalent bonds (1). The E. coli dihydrolipoyl transacetylase, the E. coli dihydrolipoyl transsuccinylase and the porcine heart dihydrolipoyl transsuccinylase each consist of 24 apparently identical subunits, and the bovine kidney and heart dihydrolipoyl transacetylase consists of 60 subunits (2). The dihydrolipoyl transacylases contain lipoic acid covalently attached by an amide bond to the ε-amino group of lysyl residues. In recent investigations of structure-function relationships and reaction mechanisms in α-keto acid dehydrogenase complexes, questions have arisen as to the exact stoichiometry of lipoyl moieties in the dihydrolipoyl transacylases. Determination of sites in the α-keto acid dehydrogenase complexes that undergo reductive acylation in the presence of radioactive α-keto acid and thiamin diphosphate and that undergo α-keto acid-dependent reaction

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Abbreviations used: GC, gas chromatography; MS, mass spectrometry.

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with radioactive N-ethylmaleimide indicates that the E. coli (3-6) and mammalian (7,8) dihydrolipoyl transacetylases contain two lipoyl moieties per polypeptide chain and that the E. coli dihydrolipoyl transsuccinylase contains only one lipoyl moiety per chain (5,9). Radioactivity measurements on the pyruvate dehydrogenase complex isolated from E. coli cells grown in the presence of [^{35}S]sulfate have led to the conclusion that each dihydrolipoyl transacetylase chain probably bears at least three lipoyl moieties (10).

Establishing the exact stoichiometry of covalently-bound lipoic acid in the α-keto acid dehydrogenase complexes is crucial to understanding its mechanism of action. Recently, a GC-MS method was developed for identification of lipoic acid in tissues (11). The method consists of acid hydrolysis of the tissue to release protein-bound lipoic acid, extraction of the lipoic acid and subsequent conversion to methyl 6,8-bis(benzylthio)octanoate for GC-MS analysis. This procedure has been adapted to the quantitative determination of protein-bound lipoic acid by isotope dilution analysis using [8,8′-$^{2}H_2$]-lipoic acid. Analysis of highly purified enzyme preparations shows that the E. coli dihydrolipoyl transacetylase contains two lipoyl moieties per polypeptide chain and that the E. coli dihydrolipoyl transsuccinylase and the bovine kidney and heart dihydrolipoyl transacetylase contain only one lipoyl moiety per chain.

MATERIALS AND METHODS

The highly purified enzyme preparations were obtained by procedures described (12-14) or by modifications thereof. $LiAl^{2}H_4$ was purchased from Merck Isotope Division. Protein concentrations were determined by amino acid analysis and by refractometry in the analytical ultracentrifuge (15).

Preparation of [8,8′-$^{2}H_2$]lipoic acid. [8,8′-$^{2}H_2$]Lipoic acid was prepared from ethyl cyclohexanone-2-acetate as outlined by Segre et al. (16) with the following modifications: the ethylene ketal of ethyl cyclohexanone-2-acetate was reduced with $LiAl^{2}H_4$ instead of $LiAlH_4$, and the intermediate compounds at each step of the synthesis were purified by column chromatography instead of vacuum distillation. After two recrystallizations from hexane, the [8,8′-$^{2}H_2$]-lipoic acid had a m.p. of 61.5°C. The mass spectrum of the derivative, methyl [8,8′-$^{2}H_2$]-6,8-bis(benzylthio)octanoate, showed a ratio of intensities of the m/e 311 ion to the m/e 313 ion of 0.024.

Hydrolysis and extraction of proteins containing covalently bound lipoic acid. Protein solutions containing 2-20 mg protein/ml were hydrolyzed with methanesulfonic acid containing 3-(2-aminoethyl)indole (17). Aliquots (50-200 µl) of the protein solutions containing 10-30 nmol of lipoic acid were

added to solutions of methanesulfonic acid and 3-(2-aminoethyl)indole of a sufficient concentration to give a final volume of 0.8 ml which was 4 M in methanesulfonic acid and 0.4% in 3-(2-aminoethyl)indole. To each sample was added 10.0 µl of a 2.42 mM solution of [8,8'-$^2H_2$]lipoic acid in 0.01 M sodium bicarbonate, and the samples were hydrolyzed at 120°C in evacuated, sealed tubes. At given times, the samples were cooled and extracted three times with methylene chloride.

Modification of lipoic acid and GC-MS determination of the isotope ratios. The methylene chloride extracts were combined, evaporated, and the residue was dissolved in 0.5 ml water, 0.5 ml of saturated sodium bicarbonate and 1 ml of methanol. To the solution was added 30 mg of sodium borohydride and 10 µl of benzyl chloride, and the mixture was kept at 60°C for 30 min. The solution was cooled, 1 ml of 6 M HCl was added slowly, and the mixture was extracted three times with methylene chloride. To the combined extracts was added an excess of diazomethane in ether. After 10 min the solution was evaporated to dryness, the residue was dissolved in 20 µl of methylene chloride, and 3-µl portions of this solution were subjected to GC-MS analysis. The operating conditions for this analysis and the mass spectrum of methyl 6,8-bis(benzylthio)-octanoate were reported previously (11).

Ion intensities were measured from the M-91 ions at m/e 311 for the unlabeled derivative and m/e 313 for the dideuterated derivative. Ten evenly spaced oscillograph traces of the m/e 311 and m/e 313 ions were obtained as the peak eluted into the mass spectrometer. The intensities for each of these ions were measured and corrected for any small column bleed ions. This method of summing the ion intensities over the entire GC peak was required to correct for a slight isotope fractionation which occurs between the labeled and unlabeled compounds during gas chromatography. The mole ratio of nondeuterated to deuterated lipoic acids was obtained from Equation 1.

$$\frac{\text{moles of nondeuterated lipoic acid}}{\text{moles of dideuterated lipoic acid}} = \frac{100N-a}{100-Nb} \qquad (1)$$

N is the observed ratio of the sum of intensities of the m/e 311 ions to the sum of intensities of the m/e 313 ions; a is the intensity of the m/e 311 ion expressed as the percent of the m/e 313 ion determined from the mass spectrum of the dideuterated derivative; b is the intensity of the m/e 313 ion expressed as the percent of the m/e 311 ion determined from the mass spectrum of the unlabeled derivative. The values for a and b were found to be 2.4% and 11.75%, respectively.

RESULTS AND DISCUSSION

Figure 1 shows the data obtained from the analysis of four known samples of lipoic acid by the procedures described in Methods. The straight line represents the expected molar ratios obtained from the isotope ratio number N and Equation 1. Repeat analysis of each sample showed a precision of better than 2% in the GC-MS measurement of the isotope ratios.

It is obvious that analysis of protein-bound lipoic acid by isotope dilution requires that the lipoic acid be released from the protein by hydrolysis quantitatively and without extensive destruction. Several different acids were examined. Methanesulfonic acid containing 3-(2-aminoethyl)indole was found to be the most useful. Isotope dilution analysis of known samples

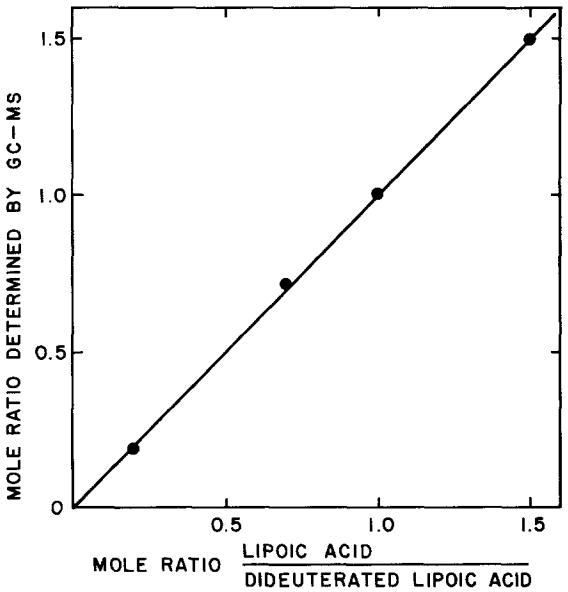

Fig. 1. Isotope ratio analyses of known samples of lipoic acid. Four known samples of lipoic acid (1-75 μg) were carried through the procedures described in Methods. Isotope ratios were calculated from intensities of the m/e 311 and m/e 313 ions. Multiple analyses (2 or 3) of each sample showed a precision of better than 2% in the GC-MS measurement of isotope ratios.

of lipoic acid subjected to hydrolysis for 5 h as described in Methods showed that 74% of the added lipoic acid was recovered. The time course of release of lipoic acid from a sample of the E. coli dihydrolipoyl transsuccinylase showed that 58.3% of the lipoic acid was released in 1 h, 98.5% in 4 h, and 100% in 24 h. Similarly, analysis of lipoic acid released from bovine kidney dihydrolipoyl transacetylase and from E. coli dihydrolipoyl transacetylase showed no significant increase after 5 h of hydrolysis.

The data in Table I demonstrate that each polypeptide chain of the E. coli dihydrolipoyl transacetylase contains two covalently bound lipoyl moieties. This finding provides strong evidence that the 48 sites on the native transacetylase (2 sites per polypeptide chain) that undergo (i) reductive acetylation in the presence of [2-^{14}C]pyruvate, thiamin diphosphate and pyruvate dehydrogenase, (ii) acetylation by [1-^{14}C]acetyl-CoA in the presence of NADH and dihydrolipoyl dehydrogenase, and (iii) reaction with N-ethyl[2,3-^{14}C]maleimide in the presence of pyruvate, thiamin diphosphate and pyruvate dehydrogenase are

Table I
Lipoic Acid Content of Dihydrolipoyl Transacylases

Enzyme	Lipoic acid content[a] mol/mg protein	mol/mol subunit[b]
E. coli dihydrolipoyl transsuccinylase	25.8	1.08
	25.8	1.08
	22.6[c]	0.95
	23.6[c]	0.98
Bovine kidney dihydrolipoyl transacetylase	19.7	1.02
	20.0	1.04
	19.7[d]	1.03
	20.1[d]	1.05
Bovine heart dihydrolipoyl transacetylase	21.4	1.11
	21.7	1.12
E. coli dihydrolipoyl transacetylase	30.7	1.98
	30.8[c]	1.99
E. coli pyruvate dehydrogenase complex	10.3[e]	1.97
	10.2	1.95
	10.8[e]	2.07

[a]Results are for a 5 h hydrolysis unless specified otherwise. Each hydrolysate was analyzed in duplicate by GC-MS unless indicated otherwise.
[b]The M_r values used are: E. coli transsuccinylase subunit, 42,000 (14); bovine heart and kidney transacetylase subunit, 52,000 (18); E. coli transacetylase subunit, 64,500 (19,20); E. coli pyruvate dehydrogenase complex, 4,600,000, containing 24 transacetylase subunits (19).
[c]Hydrolysis was for 24 h at 120°C.
[d]Hydrolysis was for 16 h at 120°C.
[e]Two different preparations of the complex were analyzed.

indeed lipoyl moieties (3-6). The data render unlikely any possibility that each transacetylase chain bears at least three lipoyl moieties (10).

In contrast to the E. coli dihydrolipoyl transacetylase, the E. coli dihydrolipoyl transsuccinylase and the bovine kidney and heart dihydrolipoyl transacetylase contain only one lipoyl moiety per polypeptide chain. This finding is consistent with previous reports that the E. coli dihydrolipoyl transsuccinylase contains 24 sites (1 per polypeptide chain) that undergo reductive succinylation in the presence of α-[5-^{14}C]ketoglutarate, thiamin diphosphate and α-ketoglutarate dehydrogenase and reaction with ^{14}C- or ^{3}H-labeled N-ethylmaleimide in the presence of α-ketoglutarate, thiamin diphosphate and α-ketoglutarate dehydrogenase (5,9). The mammalian dihydrolipoyl

transacetylase contains a set of 60 sites (1 per polypeptide chain) that undergo rapid reductive acetylation in the presence of [2-^{14}C]pyruvate, thiamin diphosphate and pyruvate dehydrogenase, and a second set of sites, possibly as many as 60, that undergo a slow reductive acetylation (7,8). Both sets of protein-bound acetyl groups are acid-stable and performic acid-labile, indicating that the acetyl groups are linked to sulfhydryl groups. The present demonstration that there is only one lipoyl moiety per transacetylase chain raises a question as to the nature and the function, if any, of the second set of acetyl-acceptor sites. A possible secondary acetylation site is one of the 4-5 half-cystine residues present in the mammalian transacetylase subunit (18).

The subunit structure and organization of the dihydrolipoyl transacetylase and the dihydrolipoyl transsuccinylase from E. coli are similar (21), and the catalytic mechanisms of the pyruvate and α-ketoglutarate dehydrogenase complexes are similar, if not identical. In view of these similarities, the fact that the E. coli dihydrolipoyl transsuccinylase and the mammalian dihydrolipoyl transacetylase contain only one lipoyl moiety per polypeptide chain, whereas the E. coli dihydrolipoyl transacetylase contains two lipoyl moieties per chain, raises a question as to the function of the second set of lipoyl moieties in the E. coli dihydrolipoyl transacetylase. It should be noted that although all 48 lipoyl moieties in the E. coli pyruvate dehydrogenase complex can be reductively acetylated in the presence of pyruvate and thiamin diphosphate, about one-half of the lipoyl moieties are apparently not essential for the overall catalytic reaction (20,22).

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