Oxygen Exchange between Acetate and the Catalytic Glutamate Residue in Glutaconate CoA-transferase from *Acidaminococcus fermentans*

**IMPLICATIONS FOR THE MECHANISM OF CoA-ESTER HYDROLYSIS**

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The exchange of oxygen atoms between acetate, glutaryl-CoA, and the catalytic glutamate residue in glutaconate CoA-transferase from *Acidaminococcus fermentans* was analyzed using $^{18}\text{O}_2$-acetate together with matrix-assisted laser desorption/ionization time of flight mass spectrometry of an appropriate undeuterated. The exchange reaction was shown to be site-specific, reversible, and required both glutaryl-CoA and $^{18}\text{O}_2$-acetate. The observed exchange is in agreement with the formation of a mixed anhydride intermediate between the enzyme and acetate. In contrast, with a mutant enzyme, which was converted to a thiol ester hydrolyase by replacement of the catalytic glutamate residue by aspartate, no $^{18}\text{O}$ uptake from H$_2^{18}$O into the carboxylate was detectable. This result is in accord with a mechanism in which the carboxylate of aspartate acts as a general base in activating a water molecule for hydrolysis of the thiol ester intermediate. This mechanism is further supported by the finding of a significant hydrolyase activity of the wild-type enzyme using acetyl-CoA as substrate, whereas glutaryl-CoA is not hydrolyzed. The small acetate molecule in the substrate binding pocket may activate a water molecule for hydrolysis of the nearby enzyme-CoA thiol ester.

The strict anaerobic bacterium *Acidaminococcus fermentans* is able to grow with glutamate as the sole source of energy. The fermentation of glutamate proceeds via the hydroxylglutarate pathway yielding ammonia, carbon dioxide, acetate, butyrate, and hydrogen as products (1). With the exception of the oxidation of glutamate to 2-oxoglutarate followed by reduction to (R)-2-hydroxyglutarate, almost all other transformations in this pathway are carried out on the CoA-ester level (2). Hence the activation of (R)-2-hydroxyglutarate is a key step in this metabolic pathway (3).

$$(\text{R})$-2$\text{Hydroxyglutarate}^{2+} + \text{acetate-CoA} \rightleftharpoons (\text{R})$-2$\text{Hydroxyglutaryl-CoA}^{-} + \text{acetate}^{-} \quad (\text{Eq. 1})$$

The transfer of coenzyme A from acetyl-CoA to (R)-2-hydroxyglutarate (Equation 1) is catalyzed by (E)-glutaconate: acetyl-CoA CoA-transferase (EC 2.8.3.12, further referred to as glutaconate CoA-transferase), which can also use propionate and glutarate as substrates. The enzyme has been purified, and the two encoding genes have been cloned, sequenced, and over-expressed in *Escherichia coli*. The hetero-octameric protein $\alpha_{4}\beta_{4}$ consists of two different subunits with molecular mass values of 35.5 kDa ($\alpha$-subunit) and 29 kDa ($\beta$-subunit) (4, 5).

The catalytic action of CoA transferases has been suggested to proceed via a mechanism outlined in Fig. 1 (6, 7). By nucleophilic attack of a glutamate residue of the enzyme at the carboxyl of the donor acetyl-CoA, a mixed anhydride between the enzyme and acetate is formed. The transiently liberated CoA$^-$ anion reattacks the glutamate carboxyl carbon to form the product acetate and an enzyme-CoA thiol ester. The second product, (R)-2-hydroxyglutaryl-CoA, is formed by repetition of the steps outlined above. It should be noted that during one catalytic cycle, one oxygen atom is transferred from the acetate to the glutamate residue. Consecutive turnovers lead to a complete equilibrium of all oxygen atoms involved in the reaction.

The catalytic glutamate residue in glutaconate CoA-transferase has been identified as amino acid 54 of the smaller $\beta$-subunit (5Glu-54). The thiol ester between coenzyme A and the glutamate residue has been reduced with sodium borohydride to the corresponding alcohol, which was identified as 2-amino-5-hydroxy-5$H$-valeric acid within a tryptic peptide (4, 5). The enzyme was crystallized, and its structure has been solved recently at 2.5 Å resolution. The crystallographic data (8) as well as sequence alignments (9) also confirmed that glutamate $\beta$54 is the catalytic residue in glutaconate CoA-transferase.

The important role of glutamate $\beta$54 has been demonstrated by site-directed mutagenesis experiments (10). Whereas the replacement of glutamate $\beta$54 by alanine ($\beta$54A) or asparagine ($\beta$54N) completely abolished the transferase activity, the glutamine ($\beta$54Q) mutant retained a remarkable residual activity (1% of the wild type). By incubating this enzyme with both substrates for 20 h at room temperature, the glutamine was completely converted to glutamate yielding a fully active CoA-transferase. Changing glutamate 54 to aspartate converted the enzyme to a thiol ester hydrolyase (9). It has been suggested that the missing methylene group in the side chain of aspartate as compared with glutamate allows a water molecule to occupy the space between the substrate and the carboxylate of aspartate $\beta$54. Activated by the carboxylate of aspartate as the general base, this water molecule should be able for a nucleophilic attack at the thiol ester carbonyl carbon and cleavage of the thiol ester bond. Alternatively, a mechanism requiring the transient formation of a mixed anhydride between aspartate and the donor carboxylic acid might be possible.

The exchange of oxygen between the CoA donor, glutaryl-CoA, the acceptor, $^{18}\text{O}_2$-acetate, and the catalytic glutamate...
FIG. 1. Reaction scheme of coenzyme A transfer by glutaconate CoA-transferase. The oxygen exchanged between acetate, glutaryl-CoA, and the catalytic glutamate residue of the enzyme (E) is shown in bold letters. It should be noted that upon several cycles of catalysis, both labeled oxygens are distributed equally among all seven participating oxygen positions in the enzyme and the substrates/products. To simplify the presentation, the equilibrium reactions are shown in a single direction.

Experimental Procedures

Materials—[18O]Water (purity 98%) was purchased from Promochem (Wesel, Germany). TPCK-treated trypsin (EC 3.4.21.4), endoproteinase Asp-N (EC 3.4.24.33), carboxypeptidase Y (EC 3.4.16.1), and coenzyme A (trillithium salt) were from Roche Molecular Biochemicals. All other chemicals were of the highest available grade and were purchased from Fluka (Buchs, Switzerland), Sigma, or Merck. Glutaconate CoA-transferase (wild type and βE54D mutant) were purified from overproducing E. coli strains as described earlier (5). The crystallization step, however, was replaced by chromatography on Q-Sepharose. The molar concentrations of the hetero-octameric enzyme were calculated with the molecular mass of the heterodimeric subunit (65 kDa).

Synthesis of Acetyl-, Glutaryl-, and Propionyl-CoA—The CoA thiol esters of acetic, glutaric, and propionic acid were prepared from the corresponding anhydrides and CoASH by the method of Simon and Shemin (11). The acetyl-CoA was desalted using Sep-Pak™ C18 cartridges (Millipore).

Synthesis of [18O]Acetate—Ethyl acetate (1 mmol) was hydrolyzed for 2 h at 50 °C in 100 μl of [18O]water saturated with gaseous hydrochloric acid. Initially formed ethyl acetate was hydrolyzed in 100 μl of [18O]water adjusted to 1 μ NaOH with 3% (w/w) sodium amalgam at 50 °C for 16 h. The solution was adjusted to pH 8 with sodium hydrogen carbonate, lyophilized, and redissolved in [18O]water. The acetate concentration was determined enzymatically (12). The 18O content of the sodium acetate (99%) was determined by gas chromatography/mass spectrometry analysis of the methyl ester synthesized with diazomethane.

Kinetics of the Oxygen Exchange—Glutaconate CoA-transferase (10 μg/ml) was added to a mixture containing 100 mM sodium phosphate, pH 7.0, 1 mM glutaryl-CoA, and 1 mM sodium [18O]acetate. Before the addition of the enzyme and after various time intervals, aliquots were taken and stopped by the addition of 1 volume of 8 M guanidinium hydrochloride, pH 7.5. The samples were applied on LiChroCART columns (4 × 250 mm, 5 μm, Merck) equilibrated with 0.1% (v/v) trifluoroacetic acid. The CoA derivatives were separated by a linear gradient from 10 to 20% (v/v) acetonitrile for 20 min at 20 °C and monitored at 260 nm. The eluting CoA derivatives were collected and analyzed by MALDI-TOF MS (see below). The CoA derivatives were quantified using an internal calibration.

Labeling of Glutaconate CoA-transferase with [18O]Acetate—The enzyme (2 nmol) was incubated for 2 min at 37 °C in 200 μl of 100 mM sodium phosphate, pH 7.0, 1 mM glutaryl-CoA, and 1 mM sodium [18O]acetate. The reaction was stopped by the addition of 1 volume of 8 M guanidinium hydrochloride, pH 7.5, and applied to reverse-phase HPLC as described. A second sample was incubated as described and separated from low molecular mass compounds by size exclusion chromatography using a Sephadex-G25 column (NAP-10, Amersham Pharmacia Biotech) equilibrated with 100 mM sodium phosphate, pH 7.0, and 100 mM [18O]acetate. Glutaryl-CoA was added to the enzyme at a final concentration of 1 mM and incubated for another 2 min at 37 °C.

Chemical Hydrolysis of the Enzyme-CoA Thiol Ester Intermediate in [18O]Water—Glutaconate CoA-transferase (2 nmol) was incubated for 1 min at 37 °C with 1 mM glutaryl-CoA in 200 μl of 100 mM sodium phosphate, pH 7.0. The reaction products were applied to reverse-phase HPLC as described below. The β-subunit of glutaconate CoA-transferase was lyophilized, redissolved in 25 μl of 4 mM guanidinium hydrochloride, 400 mM Tris-HCl, pH 8.6, in [18O]water, and incubated for 1 h at 50 °C. The mixture was diluted to 1 ml with [18O]water, lyophilized, and reductively carboxymethylated (13).

Incubation of Glutaconate CoA-transferase in [18O]-Enriched Water—The enzyme (2 nmol) was incubated at 37 °C in 200 μl of 100 mM sodium phosphate, pH 7.0, 1 mM acetyl-CoA in the presence of 50% (v/v) [18O]water for 5 (βE54D) or 240 min (wild type).

Hydroporphic Interaction HPLC Separation of CoA Derivatives and Protein Subunits—The samples were applied on a Supelcosil LC-3DP column (4.6 × 250 mm, Sigma) equilibrated with 0.1% (v/v) trifluoroacetic acid and 67% (v/v) acetonitrile on the sample slide and air-dried. Peptide samples (0.5–3 μg) were mixed 1:1 with 110 mM sinapinic acid [(Wesel, Germany). TPCK-treated trypsin (EC 3.4.21.4), endoproteinase Asp-N—The

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1 The abbreviations used are: MALDI-TOF MS, matrix-assisted laser desorption/ionization time of flight mass spectrometry; TPCK, 1,1-tosylamido-2-phenylethyl chloromethyl ketone; HPLC, high pressure liquid chromatography.
tryptic peptide β6 was lyophilized, redissolved in 100 µl of 1 M guanidinium hydrochloride, 100 mM Tris-Cl, pH 7.5. Endoproteinase Asp-N (100 ng) was added, and the digestion was allowed to proceed for 16 h at 37 °C. The resulting peptides were separated by reverse-phase HPLC as described above.

Carboxyl-terminal Sequencing—The peptide β6A was dissolved in 20 mM sodium acetate, pH 6.6, yielding a final concentration of approximately 10 µM. The peptide solution (2 µl) was mixed with 2 µl of carboxypeptidase Y (0.1 mg/ml) and incubated for 20 min at 25 °C. The reaction was stopped by the addition of 2 µl of 10% (v/v) trifluoroacetic acid. The individual molecular mass values of the generated peptides were determined by MALDI-TOF MS as described above.

Determination of the Hydrolyase Activity of Glutaconate CoA-transferase—The enzyme (0.77 nmol) was incubated at 25 °C in 1 ml of 100 mM potassium phosphate, pH 7.0, 100 mM NaCl, 1 mM 5,5′-dithio-bis-(2-nitrobenzoinate), and 200 µM acyl-CoA in either the presence or the absence of 100 mM sodium acetate. The change in absorbance at 412 nm was recorded for 10 min.

Calculation of the 18O Content from Mass Spectra—The natural isotopic distribution of large molecules such as acyl-CoA's and peptides was calculated solving the binomial equation for the isotopic distribution. This calculation was aided by the isotope pattern calculator provided by the University of Sheffield (UK) at the Sheffield ChemPuter web site (http://www.shef.ac.uk/chemistry/chemputer/isotopes.html). The 18O overlay was simulated by solving the binomial coefficient for different numbers of exchange sites. The distribution pattern for the enrichment of 18O is given by the general formula,

\[ 1 = (a + b)^n \]  
(Eq. 2)

\[ n = 1: \quad 1 = a + b \quad \text{(for acetyl-CoA)} \]  
(Eq. 3)

\[ n = 2: \quad 1 = a^2 + 2ab + b^2 \]  
(for the peptide-bound carboxylate of glutamate or aspartate)  
(Eq. 4)

\[ n = 3: \quad 1 = a^3 + 3a^2b + 3ab^2 + b^3 \]  
(for glutaryl-CoA)  
(Eq. 5)

Within these formulas, \( a^x \) represents the unchanged component of the distribution, whereas \( 2ab \) represents the \(^{18}O^{16}O \) fraction and \( b^3 \) the \(^{16}O^{18}O \) fraction of the composite spectrum in a peptide, respectively. Hence, each term in the expression relates the contribution of a particular oxygen species to the overall distribution. Because the mass difference between \(^{16}O\) and \(^{18}O\) is 2, each term except the non-labeled fraction in the above equation requires a shift of 2 on the \( m/z \) axis per exchanged oxygen. These fractional distributions are summed up to the composite distribution. The small error introduced in the natural distribution by the replacement of a relatively small number of oxygens by \(^{18}O\) is negligible within the experimental accuracy.

The measured mass spectra were integrated, and the measured distributions were fitted to simulated stick spectra minimizing the least squares deviation between the intensities of measured and simulated distributions. These evaluations were performed using the “Solve” facilities provided by Excel 97. The experimental errors of the measurements were determined by doubling the value of \( \chi^2 \) for the nonlinear least square fits.

**RESULTS**

The time course of the oxygen exchange was investigated with catalytic amounts (0.1 µM) of glutamate-CoA-transferase and initial concentrations of 1 mM for \(^{18}O\)acetate and glutaryl-CoA. At various time intervals, samples were subjected to HPLC analysis and subsequent MALDI-TOF MS of the CoA derivatives. As evident from the concentrations of acetyl-CoA and glutaryl-CoA (Fig. 2C), a chemical equilibrium of the reaction was reached after approximately 15 min with \( K_{eq} = 0.64 \pm 0.04 \). This value is close to 0.77, which can be calculated from the pK values of glutarate (pK₁ = 4.34) and acetate (pK = 4.75). In addition to acetyl- and glutaryl-CoA, increasing amounts of free CoA were detected by HPLC, whereas neither acetyl-CoA nor glutaryl-CoA exhibited significant rates of hydrolysis in control experiments.

As demonstrated in Fig. 2, the mass spectra of acetyl-CoA (Fig. 2A) and glutaryl-CoA (Fig. 2B) showed clearly resolved single isotopic peaks. The mass spectra were integrated, and the relative signal intensities for the isotopic distribution were
calculated. Alignments of these distributions to simulations allowed the determination of the $^{18}$O content of the acyl-CoAs. Minimizing the least squares deviation between simulation and measured data, the $^{18}$O content of the analytes could be calculated with an experimental error of less than 4% (see insets in Fig. 2, A and B). The isotopic equilibrium, however, was reached much more slowly than the chemical equilibrium after approximately 120 min. The $^{18}$O content at equilibrium for acetyl-CoA (45 ± 3%) and glutaryl-CoA (41 ± 3%) agreed with the calculated value of 39.4%.

To analyze the incorporation of $^{18}$O into the catalytic glutamate residue βGlu-54 of glutaconate CoA-transferase, 20 μM of enzyme was incubated with 1 mM unlabeled glutaryl-CoA and 1 mM $^{18}$O$_2$acetate for 5 min, followed by HPLC separation (Fig. 3). The $^{18}$O label of the isolated acetyl-CoA (44 ± 3%) and glutaryl-CoA (43 ± 3%), determined by MALDI-TOF MS, indicated that an isotopic equilibrium of the oxygen atoms participating in the reaction was reached.

The separated subunits of glutamate were identified by their molecular mass values obtained by MALDI-TOF MS. For the α-subunit, a molecular mass of 35,573 ± 12 Da was determined in agreement with the value of 35,568 Da predicted from the sequence (Fig. 4A). For the β-subunit, a molecular mass of 29,018 ± 18 Da (theoretical 29,017 Da) was obtained. However, a second signal at m/z 752 Da was found (Fig. 4B). In incubations with glutaryl-CoA but without acceptor carboxylate, the higher molecular mass signal was the only visible one. The mass difference observed confirmed the covalent binding of a CoA-molecule to the catalytic glutamate according to the reaction scheme shown in Fig. 1.

Neither the mass accuracy nor the resolution obtained in the spectra of the β-subunit was sufficient to determine $^{18}$O uptake. Hence, the protein was reduced with dithiothreitol, carboxymethylated, and digested with trypsin. The carboxymethylation was required to obtain the βGlu-54-containing peptide in good yields. It is well established that a significant loss of Cys-containing peptides is frequently observed if no chemical modification of the thiol group is performed. The peptide containing the catalytic glutamate residue was purified by reverse-phase HPLC on a Supelcosil LC-318 (4.6 × 250 mm, 5 μm). The monoisotopic molecular mass of that peptide (2744.6 ± 1.5 Da) was, within the experimental range of accuracy, identical to the expected mass of 2744.3 Da, but it showed a clearly visible broadening of the isotopic distribution, which was only poorly resolved (data not shown). Therefore, the peptide was digested for a second time using endoproteinase Asp-N. As shown in Fig. 5, the undecapeptide containing amino acids 48-DCHIVESGLM-58 gave well resolved mass spectra. The observed monoisotopic molecular mass of 1273.9 ± 0.5 Da was in agreement with the theoretical mass of 1274.6 Da. The natural isotopic distribution was clearly overlaid by an $^{18}$O distribution. A closer analysis of the signal distribution revealed the presence of two $^{18}$O atoms with an isotopic enrichment of 31 ± 2%. This value is significantly lower than the $^{18}$O content of the acyl-CoA derivatives and can be explained by the partial hydrolysis of the enzyme-coenzyme A thiol ester intermediate in H$_2^{18}$O. Control experiments, in which either the glutaryl-CoA or $^{18}$O$_2$acetate was omitted, showed within the experimental error no incorporation of $^{18}$O into the undecapeptide. In another control experiment, the native $^{18}$O-labeled enzyme was separated from the excess $^{18}$O$_2$acetate and subsequently incubated with 100 mM unlabeled acetate and 1 mM glutaryl-CoA. Again no $^{18}$O was found in the undecapeptide (Table I).

In addition to the peptide signals at 1274 Da, varying amounts of an oxidized form of the peptide at 1290.8 ± 1.2 Da were found. The mass difference of 16 Da suggested an oxidation of the methionine 58 to the sulfoxide. Further analysis showed that this oxidation was inevitable during the preparation of peptide samples for MALDI-TOF MS. Using a carboxy-terminal cleavage with carboxypeptidase A and MALDI-TOF MS read-out of the peptide ladder, the C-terminal sequence VESGLM of the peptide was confirmed. The oxidation site was located on methionine 58 and the $^{18}$O label of the peptide was found in glutamate 54 (data not shown). The $^{18}$O contents in the non-oxidized and the oxidized forms of the peptide were found to be identical within the experimental accuracy. However, the isotopic distribution of the oxidized form of the peptide was always slightly disturbed by the sodium adduct of the non-oxidized peptide.

As evident from Fig. 2, wild-type glutamate also catalyzed the slow hydrolysis of glutaryl-CoA. Further analysis revealed that this hydrolytic activity was significantly stimulated by 100 mM acetate (114 milliunits/mg as compared with <2 milliunits/mg, respectively), whereas the hydrolysis of acetyl-CoA was not further enhanced (105 milliunits/mg as compared with 98 milliunits/mg). Propionyl-CoA was hydrolyzed more slowly than acetyl-CoA, and a stimulation by 100 mM acetate was found (54 and 118 milliunits/mg, respectively) (Table II). It should be noted that the apparent $K_m$ values observed for the hydrolysis of different acyl-CoA derivatives were about 500 times lower (67–69 μM, Table II) than the apparent $K_m$ value for acetate (26 mM) in the CoA-transferase reaction in the presence of 100 μM glutaryl-CoA (4).

In this paper, we have demonstrated that a mechanism-based introduction of $^{18}$O label can be followed during several steps of analysis to the level of the labeled amino acid and that quantitative results can be obtained from the integrated spectra. These results encouraged us to investigate the hydrolysis reaction catalyzed by wild-type glutamate transferase. As expected, the chemical hydrolysis of the HPLC-purified thiol ester between βGlu-54 and CoA in H$_2^{18}$O at pH 8.6 led to the incorporation of almost exactly 1.0 $^{18}$O into the catalytic glutamate residue (47 ± 3%, Table I). By enzymatic hydrolysis of
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acetyl-CoA in 50% H₂¹⁸O catalyzed by wild-type glutaconate CoA-transferase, both the remaining acetyl-CoA (0.85 mM) and the βGlu-54 residue became equally labeled (14 ± 3% and 12 ± 3%, respectively (Table I).

A glutaconate CoA-transferase, in which the catalytic glutamate has been replaced by aspartate by site-directed mutagenesis, has been shown to be a thiol ester hydrolyase rather than a coenzyme A-transferase. Although the replacement of glutamate by aspartate is predicted to generate an additional cleavage site for endoproteinase Asp-N, this new cleavage site was apparently not used under the experimental conditions. If this enzyme was incubated in the presence of either [¹⁸O]acetate and glutaryl-CoA or glutaryl-CoA in H₂¹⁸O, no ¹⁸O was found in the corresponding undecapeptide even after complete hydrolysis of glutaryl-CoA. In addition, the formation of an enzyme-CoA thiol ester was never observed with this enzyme.

**DISCUSSION**

The incubation of [¹⁸O]acetate and glutaryl-CoA in the presence of glutaconate CoA-transferase from A. fermentans redistributed the ¹⁸O label, initially present only in acetate, among all seven of the participating oxygen atoms. Whereas the ¹⁸O content of the three oxygen atoms of glutaryl-CoA increased immediately, the decrease in the ¹⁸O content of the single oxygen atom of acetyl-CoA was slightly delayed. This observation is in agreement with the statistical proposal for the exchange reaction. Initially, the reaction proceeds predominantly

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**FIG. 5.** Mass spectrometry of the undecameric peptide containing the catalytic glutamate of glutaconate CoA-transferase. The HPLC-purified β-subunit of glutaconate-CoA-transferase incubated in the presence of [¹⁸O]acetate and glutaryl-CoA (A) or of the enzyme after subsequent incubation with [¹⁸O]acetate and glutaryl-CoA (B, re-exchanged sample) was in succession digested with trypsin and endoproteinase Asp-N as described under "Experimental Procedures." The undecapeptide was prepared by HPLC and analyzed MALDI-TOF MS using indole-3-carboxylic acid as matrix. The spectra were collected with an accelerating voltage of 20,000 V, 58% grid voltage, and an 80-ns delay time. The 100% intensity scale refers to 6,000 counts in B and 14,000 counts in A. In addition to the signal of the peptide (theoretical: 1275.6 Da for [M + H]+) the methionine sulfoxide form of the peptide at m/z = 1292 is visible. Note, that the signals at m + 4 and m + 5, indicating the exchange of two oxygen atoms, are clearly increased in both of the distributions in A, demonstrating the exchange of both oxygens in the glutamate carboxylate.

**TABLE I**

1⁸O labeling of the catalytic glutamate residue of wild-type glutaconate CoA-transferase and βE54D mutant

|          | Normalized relative signal intensities | ¹⁸O content (%) |
|----------|---------------------------------------|-----------------|
|          | m ±1 +2 +3 +4 +5                       |                 |
| Wild type, residue βGlu-54 | | |
| Natural isotopic distribution | 100 66 34 13 4 1 31 (±2) | |
| + glutaryl-CoA + [¹⁸O]acetate | 59 49 20 | |
| Glutaryl-CoA omitted | 100 64 24 11 5 0 <2 ²b | |
| [¹⁸O]acetate omitted | 100 66 36 11 5 0 <1 ²b | |
| + glutaryl-CoA + [¹⁸O]acetate and re-exchange with [¹⁸O]acetate | 100 65 35 18 4 0 <2 ²b | |
| Hydrolysis of enzyme CoA-thiol ester in H₂¹⁸O | 6 4 100 64 30 12 47 | |
| Hydrolysis of acetyl-CoA in H₂¹⁸O | 100 70 64 34 16 4 12 (±3) | |
| βGlu-54 mutant, residue βAsp-54 | | |
| Natural isotopic distribution | 100 64 33 13 4 1 <3 ²b | |
| + glutaryl-CoA + [¹⁸O]acetate | 100 68 41 17 6 2 | |
| Hydrolysis of acetyl-CoA in H₂¹⁸O | 100 74 31 10 3 0 <4 ²b | |

a The normalized relative signal intensities were calculated from the integrated mass spectra (n = 3–5). The value given for the monoisotopic mass (m) corresponds to the signal of the monoisotopic peak at m/z = 1275.6 Da for the wild type and at m/z = 1261.7 Da for the mutant. The following single isotopic peaks have been numbered +1 to +5. The distribution is normalized on the signal intensity of the highest peak.

b The calculated ¹⁸O content of these samples was smaller than the experimental error.

c The signal distribution of this peptide did not align to an equal exchange distribution for both carboxylate oxygen atoms. The introduction of only one oxygen, whereas hydrolysis increases the exchange to 93% and is close to the ¹⁸O content of the H₂¹⁸O (98%) used.
in the direction of acetyl-CoA formation. Hence, the initially formed acetyl-CoA is predicted to carry the same $^{18}$O content as the added $[^{18}$O$_2]$acetate. Moreover, after a few catalytic cycles, the oxygen atoms of the catalytic glutamate had almost the same $^{18}$O content as the $[^{18}$O$_2]$acetate. Hence, all glutarate molecules released from glutaryl-CoA are $^{18}$O-labeled, and the reformer glutaryl-CoA contained $^{18}$O.

The data demonstrate the exchange of oxygen between $[^{18}$O$_2]$acetate and glutarate-CoA transferase as predicted by the formation of a mixed anhydride between acetate and the catalytic glutamate residue during catalysis of CoA-transferase. The observed exchange was found to require the presence of both $[^{18}$O$_2]$acetate and glutarate-CoA and was shown to be completely reversible (Table I). The $^{18}$O label has been found to be localized on both oxygen atoms of glutarate residue 54 within the $\beta$-subunit of the enzyme, which forms the enzyme-CoA thiol ester intermediate as predicted by the mechanism outlined above (Fig. 1). Only one oxygen atom of the catalytic glutamate residue became labeled with $^{18}$O by chemical hydrolysis of the isolated thiol ester between CoA and the wild-type enzyme in $H_2^{18}$O.

For the $E54D$ mutant of glutarate-CoA-transferase, it was proposed that because of the shortening side chain of aspartate as compared with the original glutamate residue, a water molecule might occupy the space between the aspartate and the acyl-CoA in the active center (9). Acting as a general base, the carboxylate group of aspartate has been suggested to activate water for hydrolysis of the thiol ester, which is in agreement with the absence of any $^{18}$O in the $\beta$-Asp-54 carboxylate. This result, however, cannot exclude the intermediacy of a mixed anhydride between $\beta$Asp-54 and acetate, which is specifically attacked by $H_2^{18}$O at the acetyl carbonyl (Fig. 6).

Interestingly, the slow hydrolysis of acetyl-CoA in $H_2^{18}$O catalyzed by the wild-type enzyme led to a significant $^{18}$O incorporation into the $\beta$Glu-54 carboxylate. In this case, at least three mechanisms are possible that cannot be distinguished because of the subsequent much faster equilibration of the four oxygen atoms of acetate and $\beta$Glu-54 by CoA-transferase. The three mechanisms are as follows: (i) hydrolysis of acetyl-CoA by assistance of $\beta$Glu-54 carboxylate; (ii) hydrolysis of either carboxyl of the mixed anhydride; or most likely, (iii) the acetate catalyzed hydrolysis of the enzyme-thiol ester.

Although the low but significant CoA-ester hydrolyase activity has been recognized much earlier (4), this current work shows that hydrolysis is due to an intrinsic property of the enzyme and subject to catalysis by acetate. Whereas glutaryl-CoA was not hydrolyzed ($<2$ milliunits/mg), hydrolyase activity was observed (114 milliunits/mg) in the presence of 100 mM acetate exhibiting an apparent $K_m = 69 \mu$M acetate (Table II).

Further analysis revealed that acetyl-CoA is hydrolyzed at the same rate and is not affected by the addition of acetate, whereas propionyl-CoA behaves in a manner in between acetyl- and glutaryl-CoA. The low apparent $K_m$ for acetate observed in the hydrolysis of acyl-CoA substrates is comparable with the low apparent $K_m$-values observed in CoA-ester hydrolysis by the $E54D$ mutant and for the specific acetyl-CoA-dependent exchange of 1.0 $^3$H from $[2,4-^3$H]glutaconate with the solvent water (14). These low $K_m$ values show that the enzyme is able to bind these substrates very tightly. In all three reactions no complete CoA-transfer occurred. The unusually high $K_m$-values

### Table II

| Acyl-CoA         | Control | $>100$ mM sodium acetate | Apparent $K_m$ for acetate |
|------------------|---------|--------------------------|---------------------------|
|                   | milliunits/mg | milliunits/mg | $\mu$M |
| Wild-type        |         |                         |                           |
| Acetyl-CoA       | 105     | 95                       | 67                        |
| Propionyl-CoA    | 54      | 118                      | 67                        |
| Glutarate-CoA    | <2      | 114                      | 69                        |
| $E54D$ mutant    | 15,000  |                         |                           |
| Glutarate-CoA    |         |                         |                           |

**Fig. 6.** Asymmetric cleavage of a mixed anhydride in the presence of $H_2^{18}$O. Within a sterically hindered environment as the active center of an enzyme, the hydrolysis of a mixed anhydride between the catalytic residue and acetic acid may occur asymmetrically. The $^{18}$O label will be present in the enzyme only in the first case (left), whereas it will be released as $[^{18}$O]acetate in the second case (right).

**Fig. 7.** Activation of a water molecule for hydrolysis of enzyme-CoA thiol ester by wild-type glutarate-CoA-transferase. Whereas the binding of glutarate to a tight fitting dicarboxylate binding site does not allow the entry of an additional water molecule (I), the binding of acetate (or propionate) to a second dilated monocarboxylate binding site may allow entry and activation of a water molecule for hydrolysis of enzyme-CoA thiol ester intermediate (II). Hence, hydrolysis of the enzyme-CoA thiol ester and transfer of CoA from the enzyme to acetate are competitive reactions.
in the complete CoA-transfer are in accordance with the hypothesis of White and Jencks (7) that CoA-transferases are able to convert binding energy into rate enhancement. If one adds the ~370-fold increase in $K_m$ for acetate and the ~170-fold increase in $K_m$ for acetyl-CoA (14), the transition state could be lowered by 27 kJ/mol.

It has been shown by reduction with NaBH$_4$ (4) and by MALDI-TOF MS (this paper) that incubation of the CoA-transferase with glutaryl-CoA alone results in up to 100% conversion of the enzyme into the stable CoA-ester. Addition of acetate results in two reactions, CoA-transfer and hydrolysis. If hydrolysis requires a proceeding CoA-transfer, then no difference should be observed in the $K_m$ for both processes. Hence, it appears likely that acetate stimulates the hydrolysis of the enzyme CoA-ester intermediate by activating a water molecule. One of the possible two carboxylate binding sites should be large enough to accommodate water together with the general base acetate or, to a lesser extent, with propionate (Fig. 7). The hydrolysis at the stage of the enzyme-CoA ester rather than acyl-CoA is in accordance with the high reactivity of the former toward NaBH$_4$. Probably the enzyme-CoA ester is somehow distorted, which makes the carbonyl group more accessible for nucleophiles.

In E. coli 3-methyladenine-DNA glycosylase II (DNA glycosidase, AlkA) the catalytic aspartate residue 238 of the enzyme was proposed to activate a water molecule for hydrolysis of the N-glycosidic bond of the substrate (15). In agreement with this mechanism, it was found that the exchange D238N by mutation completely abolished the hydrolyase activity of the protein. The same holds true for the mutants of glutarate synthesis completely abolished the hydrolyase activity of the enzyme. Whereas the protein. The same holds true for the mutants of glutarate synthesis completely abolished the hydrolyase activity of the enzyme. It was found that the exchange D238N by mutant E54D (10) results in two reactions, CoA-transfer and hydrolysis. If hydrolysis requires a proceeding CoA-transfer, then no difference should be observed in the $K_m$ for both processes. Hence, it appears likely that acetate stimulates the hydrolysis of the enzyme CoA-ester intermediate by activating a water molecule. One of the possible two carboxylate binding sites should be large enough to accommodate water together with the general base acetate or, to a lesser extent, with propionate (Fig. 7). The hydrolysis at the stage of the enzyme-CoA ester rather than acyl-CoA is in accordance with the high reactivity of the former toward NaBH$_4$. Probably the enzyme-CoA ester is somehow distorted, which makes the carbonyl group more accessible for nucleophiles.

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Taking all of these findings together, it appears far more likely that the carboxyl group of aspartate can act as a general base in the activation of a water molecule, allowing the direct hydrolysis of the acyl-CoA substrate rather than the intermediate formation of a mixed anhydride, which is further hydrolyzed.

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