Fatty acid-requiring mutant of *Saccharomyces cerevisiae* defective in acetyl-CoA carboxylase

(structural gene mutation/multifunctional enzyme/ATP affinity alteration)
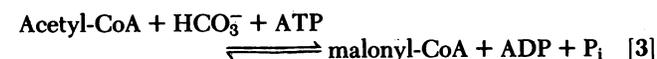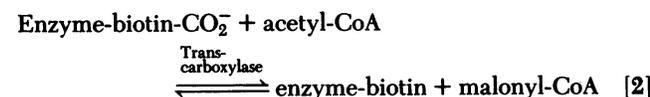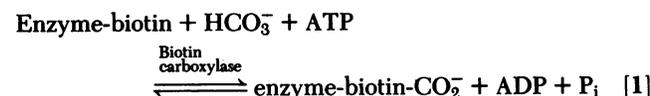
RAINER ROGGENKAMP*, SHOSAKU NUMA†, AND ECKHART SCHWEIZER*

*Lehrstuhl für Biochemie der Universität Erlangen-Nürnberg, 8520 Erlangen, West Germany; and †Department of Medical Chemistry, Kyoto University Faculty of Medicine, Kyoto 606, Japan

ABSTRACT The isolation and biochemical properties of a *Saccharomyces cerevisiae* mutant (*acc1-167*) defective in acetyl-CoA carboxylase [acetyl-CoA:carbon-dioxide ligase (ADP forming), EC 6.4.1.2] activity are described. The mutant is deficient in *de novo* biosynthesis of long-chain fatty acids and specifically requires a saturated fatty acid of chain length 14–16 C atoms for growth. Fatty acid synthetase levels were normal, but the acetyl-CoA carboxylase specific activity of the purified enzyme was reduced to approximately 5% compared to wild-type yeast. Upon sodium dodecyl sulfate/polyacrylamide gel electrophoresis the purified mutant enzyme migrated as a single band and was essentially indistinguishable from the wild-type enzyme. The study of acetyl-CoA carboxylase partial activities revealed that the biotin incorporation capacity and the transcarboxylase partial activity were unaffected whereas the biotin carboxylase component enzyme exhibited less than 10% of wild-type specific activity. This biotin carboxylase mutational deficiency could be ascribed to a more than 90% reduction of V_{max} and to a comparable increase in the K_m value for ATP, which was accompanied by an increased requirement for Mg^{2+}. It is concluded that *acc1-167* contains a structural gene mutation in the biotin carboxylase domain of acetyl-CoA carboxylase.

The first step in the pathway of biosynthesis of long-chain fatty acids is the formation of malonyl-CoA catalyzed by acetyl-CoA carboxylase [acetyl-CoA:carbon-dioxide ligase (ADP-forming), EC 6.4.1.2] (1). Overall acetyl-CoA carboxylation may be resolved into two sequential reaction steps: biotin carboxylation and carboxybiotin-acetyl-CoA transcarboxylation (eqs. 1 and 2).

Enzyme-biotin + HCO_3^- + ATP

$$\xrightarrow[\text{Biotin carboxylase}]{} \text{enzyme-biotin-}CO_2^- + \text{ADP} + P_i \quad [1]$$

Enzyme-biotin-CO_2^- + acetyl-CoA

$$\xrightarrow[\text{Trans-carboxylase}]{} \text{enzyme-biotin} + \text{malonyl-CoA} \quad [2]$$

Acetyl-CoA + HCO_3^- + ATP

$$\xrightarrow{\quad} \text{malonyl-CoA} + \text{ADP} + P_i \quad [3]$$

In accordance with this reaction mechanism, the acetyl-CoA carboxylase of prokaryotic organisms represents a multienzyme system of three distinct functional protein components specifically exhibiting biotin carboxylase, transcarboxylase, or biotin incorporation capacities (2). On the other hand, the enzymes isolated from mammalian and avian livers (3–5) and that from yeast (6, 7) seem to be multifunctional proteins with all three different functional sites being located on the same

polypeptide chain (8). Such multifunctional enzymes imply a number of interesting problems regarding their biosyntheses, molecular organization, and reaction mechanisms. Their study should be greatly facilitated by the availability of appropriate mutants. Yeast cells deficient in acetyl-CoA carboxylase are expected to require saturated long-chain fatty acids for growth. In this respect they would resemble the previously characterized fatty acid synthetase mutants, which apparently constitute the majority of fatty-acid-requiring yeast mutants (9, 10). In fact, a more detailed analysis of the fatty-acid-dependent mutants available in this laboratory revealed some candidates for acetyl-CoA carboxylase deficiency. The biochemical characteristics of one of these strains, *acc1-167*, are described in this study.

MATERIALS AND METHODS

Chemicals, Media, and Growth Conditions. The composition of the complex media YEP-FA (fatty-acid-containing) and YEP (fatty-acid-free) and the sources of most chemicals used in this study have been described (9). Fatty-acid-supplemented minimal media contained (per liter): 50 g of sucrose, 4 g of vitamin-free Casamino acids (Difco), 10 g of potassium citrate, 2 g of citric acid, 10 g of Tween 40, 0.1 g of myristic acid, 0.85 g of KCl, 0.25 g of $CaCl_2$·$2 H_2O$, 11 g of KH_2PO_4, 0.25 of g $MgSO_4$·$7 H_2O$, 5 mg of $MnSO_4$, 5 mg of $FeCl_3$, 50 mg of inositol, 5 mg of nicotinic acid, 2.5 mg of calcium pantothenate, 10 µg of thiamine·HCl, 10 µg of pyridoxine·HCl, and 15 µg of biotin. [^{14}C]Biotin (specific activity 58 Ci/mol; 1 Ci = 3.7 × 10^{10} becquerels), [8-^{14}C]ADP (specific activity 52 Ci/mol), and [1-^{14}C]acetyl-CoA (specific activity 58 Ci/mol) were purchased from Amersham-Buchler (Braunschweig). Specific acetyl-CoA carboxylase antisera were prepared by immunization of rabbits as described (11).

Yeast Strains and Isolation of Acetyl-CoA Carboxylase Mutant. The haploid *Saccharomyces cerevisiae* strain SC 167 (*α, ade1*), a descendant of the widely used S 288 C reference strain, was mutagenized with ethyl methane sulfonate (Eastman) essentially as described (9). Fatty-acid-requiring mutants were isolated according to the procedure adopted for isolation of fatty acid synthetase mutants (9). Subsequently, these mutants were screened for *fas1* and *fas2* allelism by complementation analysis with appropriate fatty acid synthetase mutants. Oleic-acid-dependent strains were excluded by their failure to grow on palmitic-acid-supplemented media. The mutants obtained after this selection were tentatively considered as acetyl-CoA carboxylase mutants. To characterize this deficiency we chose the gene symbol *acc*, which now replaces the designation *fas3* used in an earlier publication (10).

Acetyl-CoA Carboxylase Purification and Enzyme Assays. Acetyl-CoA carboxylase was purified from 1.2–1.5 kg (wet weight) of wild-type or mutant yeast cells by the procedure of Sumper and Riepertinger (12) as modified by Mishina *et al.* (7). In addition, the following alterations were introduced: cells

Table 1. Fatty acid composition of *acc1-167* after tridecanoic acid- or pentadecanoic acid-supplemented growth

Added fatty acid	Fatty acid composition, %													
	13:0	13:1	14:0	14:1	15:0	15:1	16:0	16:1	17:0	17:1	18:0	18:1	Even chain length	Odd chain length
13:0	10.2	5.5			21.4	25.5	1.7	1	8.2	25.5	Trace	0.8	3.5	96.3
15:0					39.6	32.2	0.8	1	6.8	19.6	Trace	0.8	2.6	98.2

Experimental procedures were as described (19).

were disrupted in the presence of phenylmethylsulfonyl fluoride (1 mg/ml) to reduce protease activities. Furthermore, polyethylene glycol fractionation was performed at 5–9.5% polyethylene glycol (average M_r 3000). With the exception of one wild-type enzyme preparation used for rabbit immunization, the final hydroxyapatite chromatography was omitted. Instead, a 2-fold larger DEAE-cellulose column than that indicated by Mishina *et al.* (7) was used. From this chromatography, all fractions containing more than 50% of the peak fraction activity were pooled and concentrated by reversed dialysis (8 hr against saturated ammonium sulfate, pH 7.2, containing 5 mM 2-mercaptoethanol and 1 mM EDTA). This procedure, when followed by hydroxyapatite chromatography, allowed one to isolate 25 mg of purified enzyme (specific activity 4900 milliunits/mg) starting from 1.5 kg of yeast cells.

For the isolation of mutant acetyl-CoA carboxylase, the cells were grown on fatty-acid-containing synthetic medium in the presence of 0.1 μM [^{14}C]biotin. After the second ammonium sulfate precipitation, the radioactively labeled enzyme preparation was mixed with the corresponding fraction obtained from twice the amount of unlabeled cells. Purification of the mutant enzyme was followed by testing for its residual acetyl-CoA carboxylase activity and its [^{14}C]biotin content.

For the purpose of enzyme isolation, yeast cells were grown in a 70-liter fermenter (Chemap, Ratingen, West Germany) in YEP-FA medium at 30°C under vigorous aeration up to late exponential phase. After harvesting, the mutant cultures were routinely screened for revertants by replica plating. Usually, the frequency of revertants was less than 1/10⁵ cells. Acetyl-CoA carboxylase activity was determined unless otherwise indicated by the combined spectrophotometric assay with fatty acid synthetase at 25°C (13). The reverse reaction of acetyl-CoA carboxylase was measured as described by Matsuhashi *et al.* (13). Carboxylation of biotin (Eq. 1) was assayed by the [^{14}C]-ADP/ATP exchange reaction (14). The reaction was terminated by the addition of EDTA to a final concentration of 100 mM. The transcarboxylation partial reaction (Eq. 2) was assayed by the [^{14}C]acetyl-CoA/malonyl-CoA exchange reaction as described by Gregolin *et al.* (15), except that citrate was omitted from the reaction mixture. [^{14}C]Malonyl-CoA formed was determined as [^{14}C]malonic acid by scintillation spectrometry according to Patterson and Greene (16). Protein was determined by the method of Warburg and Christian (17) or Lowry *et al.* (18).

Table 2. Fatty acid synthetase and acetyl-CoA carboxylase activities from wild-type and *acc1-167* cells

Enzyme	Specific activity, milliunits/mg*		
	acc1-167	Wild type	1:1 mixture
Fatty acid synthetase†	8.0	8.4	
Acetyl-CoA carboxylase‡	2.4	42	20.8

* Samples containing equal amounts of protein were mixed immediately after cell rupture.
† Assayed spectrophotometrically in crude extracts after centrifugation at 20,000 × *g* for 30 min (20).
‡ Assayed after first ammonium sulfate fractionation.

RESULTS

Growth Dependence of *acc1-167* on Exogenous Long-Chain Fatty Acids. The mutant *acc1-167* absolutely requires a saturated long-chain fatty acid for growth. The fatty acid concentration needed for optimal growth as well as the chain length specificity of the mutant were the same as those reported earlier for fatty acid synthetase mutants (9). In agreement with these results, essentially no endogenous even-chain-length fatty acid biosynthesis was observed in *acc1-167* cells when grown on 13:0 or 15:0 fatty acids as supplements (Table 1). In addition, this experiment indicated that only *de novo* fatty acid synthesis, but not the fatty acid elongation process, was affected in the *acc1-167* mutation. The content of odd-chain-length fatty acids in wild-type cells grown under similar conditions was approximately 20% (19).

Purification and Characterization of *acc1-167* Acetyl-CoA Carboxylase. The mutant contained, if a crude enzyme preparation was tested, only about 5% of wild-type acetyl-CoA carboxylase activity, whereas the specific activity of fatty acid synthetase was unaffected (Table 2). According to immunotitration experiments, the low level of *acc1-167* acetyl-CoA carboxylase activity is due to an inactivation of the enzyme rather than to a reduction in the amount of enzyme present in the cell (Fig. 1). In these experiments, the quantity of mutant acetyl-CoA carboxylase protein was determined by immunological titration of a crude wild-type enzyme preparation (first ammonium sulfate precipitate) in the presence and absence of a corresponding fraction of mutant cells. In the presence of an equal amount, on a protein basis, of the mutant preparation,
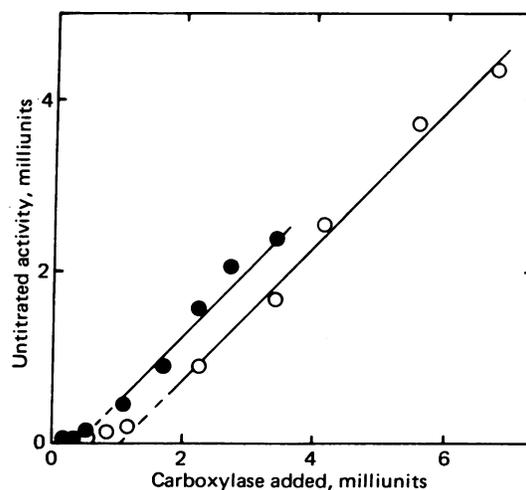

FIG. 1. Quantitative determination of *acc1-167* acetyl-CoA carboxylase protein by immunochemical titration. Wild-type and *acc1-167* cells were grown in YEP-FA medium to late exponential phase. Acetyl-CoA carboxylase was purified up to the first ammonium sulfate precipitation. Samples (50 μl) containing increasing amounts of either wild-type enzyme (O) or a 1:1 mixture, on a protein basis, of wild-type and mutant enzyme (●) containing the activities indicated were added to 10 μl of specific acetyl-CoA carboxylase antiserum. After 5 hr of incubation at 4°C, acetyl-CoA carboxylase activities were determined by the [^{14}C]bicarbonate fixation method at 25°C without removal of immunoprecipitates formed (21).
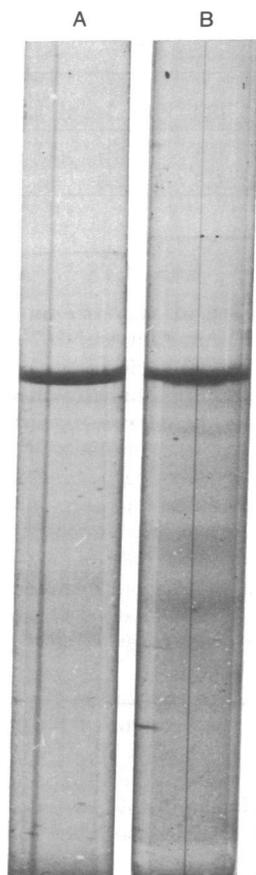
FIG. 2. Sodium dodecyl sulfate/polyacrylamide gel electrophoresis of wild-type (*B*) and *acc1-167* (*A*) acetyl-CoA carboxylase. Experimental conditions were as described (3); 8 μg of protein was applied to each gel.

Table 3. Acetyl-CoA carboxylase overall and component activities of wild-type and *acc1-167* enzymes

Enzyme function	Specific activities, milliunits/mg	
	acc1-167	Wild type
Overall acetyl-CoA carboxylation:		
Forward reaction	140	2300
Reverse reaction	<1	147
Biotin carboxylation	1.5	21
Transcarboxylation	8.3	8.2
Biotin incorporation*	354	424

Except for biotin incorporation measurements, all enzyme preparations used had been purified up to DEAE-cellulose chromatography. The acetyl CoA carboxylase biotin content was determined by immunoprecipitation of 4.0-mg samples (0.6 ml) of a crude enzyme preparation (first ammonium sulfate precipitation) with 1.5 ml of specific acetyl-CoA carboxylase antiserum as described (3). Enzyme was labeled with [^{14}C]biotin as described in *Materials and Methods*.
* Biotin incorporation is expressed as cpm/4.0-mg protein sample.

the point of intersection obtained by extrapolation of the linear portion of the titration curve shifted from 1.1 milliunits to 0.5 milliunit, as shown in Fig. 1. This shift of about 50% indicates that the content of acetyl-CoA carboxylase protein in the mutant cells is nearly equal to that in wild-type cells. Similarly, the presence of an acetyl-CoA carboxylase inhibitor in mutant cells could be excluded by the fact that wild-type enzyme activity remained unaffected by the addition of mutant cell extract (Table 2).

For acetyl-CoA carboxylase purification, up to DEAE-cellulose chromatography the same procedure was used for both the wild-type and the *acc1-167* enzymes. However, only the wild type enzyme could be successfully recovered from subsequent hydroxyapatite fractionation. In contrast, the elution profile of the mutated enzyme was greatly distorted and enzyme yields were extremely low. Nevertheless, the two carboxylases obtained after DEAE-cellulose chromatography were homogeneous and indistinguishable by sodium dodecyl sulfate/polyacrylamide gel electrophoresis. For both enzymes, the presence of only a single band of the same molecular weight in each case was observed (Fig. 2).

Component Enzyme Activities of Purified *acc1-167* Acetyl-CoA Carboxylase. With the purified *acc1-167* enzyme, overall acetyl-CoA carboxylase activity was determined for both the forward and reverse reactions. As in the crude enzyme preparation, the specific activity of the forward reaction was only approximately 5% of the wild-type enzyme, whereas that of the reverse reaction was undetectable under the experimental conditions used (Table 3). In addition, the three known com-

ponent functions of acetyl-CoA carboxylase have been studied: biotin incorporation, biotin carboxylation, and acetyl-CoA/malonyl-CoA transcarboxylation (see Eqs. 1 and 2). As indicated in Table 3, radioactively labeled biotin was bound to wild-type and *acc1-167* acetyl-CoA carboxylase to essentially the same extent. Similarly, in the purified mutant acetyl-CoA carboxylase preparations, the transcarboxylase component enzyme exhibited a wild-type-like specific activity (Table 3). The various activities of wild-type acetyl-CoA carboxylase as listed in Table 3 are, with the exception of a lower transcarboxylase activity, in their relative proportions comparable to values reported by others for the yeast enzyme (6, 13). Compared to citrate-activated liver acetyl-CoA carboxylase, however, the component activities in yeast appear to be remarkably low (15, 22). On the other hand, the specific activity of biotin carboxylase was apparently reduced to about 7% compared to wild type (Table 3). To characterize this defect further, we determined the K_m values for ATP and bicarbonate of the mutant acetyl-CoA carboxylase. As shown in Fig. 3, the mutant enzyme has a K_m for ATP about 10 times higher than that of the wild-type carboxylase, whereas that for bicarbonate is essentially the same.
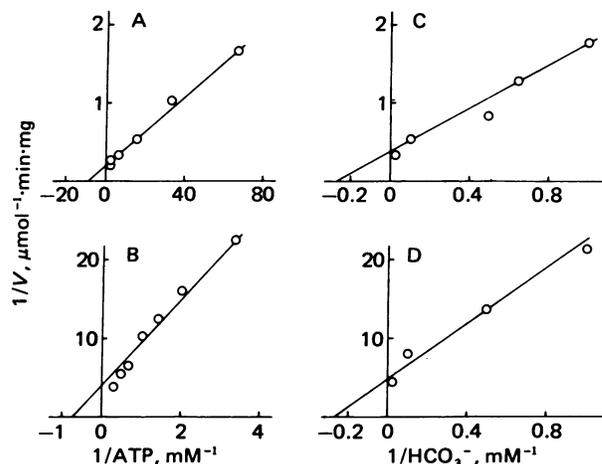

FIG. 3. Lineweaver–Burk plots of the ATP and HCO$_3^-$ dependencies of purified wild-type (*A* and *C*) and *acc1-167* (*B* and *D*) acetyl-CoA carboxylase. Mg^{2+} concentrations were 10 mM in all experiments.
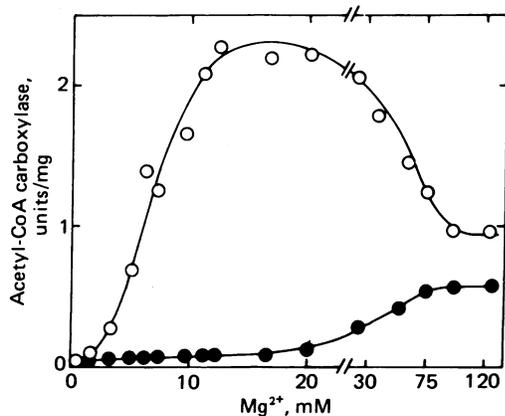
FIG. 4. Mg^{2+} requirement of wild-type (O) and *acc1-167* (●) acetyl-CoA carboxylase. ATP concentrations were 2.5 mM in all experiments.

Concomitantly, the Mg^{2+} requirement of the mutant enzyme was significantly higher than that of the wild type (Fig. 4). If both enzymes were assayed at their optimal respective Mg^{2+} concentrations, the relative specific activity of the mutant enzyme could be increased to 25% of the wild-type value. Concomitant increase of ATP concentration had no additional stimulatory effect. In addition, it may be seen from Fig. 3 that in both experiments V_{max} of the mutant enzyme was also considerably reduced compared to the wild-type carboxylase.

DISCUSSION

In the present study, a *S. cerevisiae* mutant has been characterized as being specifically deficient in acetyl-CoA carboxylase activity. As has already been described for fatty acid synthetase mutants of yeast, the strain deficient in acetyl-CoA carboxylase is incapable of synthesizing *de novo* long-chain saturated fatty acids whereas medium-chain-length fatty acid elongation apparently proceeds unimpaired. This indicates that malonyl CoA-dependent fatty acid elongation, which has been described for several other systems (23), plays essentially no major role in yeast.

Because the amount of acetyl-CoA carboxylase synthesized in the mutant cells as well as its gross protein chemical characteristics are identical to the wild-type enzyme, it is concluded that *acc1-167* represents a missense-type mutation within a crucial region of the acetyl-CoA carboxylase structural gene(s). This region apparently encodes the biotin carboxylase active site since only the specific activity of this component enzyme was significantly reduced in the mutant. The biotin carboxylase mutational defect may be ascribed partly to the greatly reduced affinity of this enzyme for ATP and Mg^{2+}. According to the common view of the involvement of Mg^{2+} in ATP-requiring reactions, it appears likely that both effects are intrinsically related (24). Due to the strong substrate inhibition observed at higher ATP concentrations, it is impossible to compensate for this defect experimentally. The kinetic characteristics of the mutated enzyme suggest that not only K_m for ATP, but also V_{max} of the mutant biotin carboxylase, is altered. In view of the fact, however, that the interaction of biotin carboxylase with bicarbonate is unaffected, it may be concluded that the mutational alteration is restricted to only a limited portion of the biotin carboxylase protein.

In agreement with earlier reports (6, 7), the purified yeast acetyl-CoA carboxylase migrated as a single band upon sodium dodecyl sulfate/polyacrylamide gel electrophoresis. From this it was concluded that it represents in yeast and other eukaryotic organisms a multifunctional protein rather that a multienzyme complex (8, 25). Further genetic and biochemical studies using additional acetyl-CoA carboxylase mutants defective in other functional components are expected to provide answers to important questions regarding the biosynthesis, structure, and function of this complex enzyme.

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