GENE-PROTEIN RELATIONSHIPS IN ACETATE-REQUIRING MUTANTS OF *NEUROSPORA CRASSA* DEFICIENT IN ACTIVITY OF PYRUVATE DEHYDROGENASE COMPLEX

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Three groups of acetate-requiring mutants of *Neurospora crassa*, *ace-2*, *ace-3* and *ace-4*, were found to lack pyruvate dehydrogenase complex (PDHC) activity. Gene-protein relationships in these mutants were investigated. All heterokaryons composed of inter-genic combinations exhibited PDHC activity. In addition, two *ace-4* mutants complemented *in vivo*. On the other hand, *in vitro* complementation of PDHC activity by simple mixing two extracts was detected only in combinations of *ace-2* + *ace-4* and *ace-3* + *ace-4*. Each of the three component enzymes of PDHC was detected as two peaks by Sephadex G-200 gel chromatography. One of them eluted from Sephadex column at void volume comprises high molecular weight aggregate. In the case of the wild-type strain, it may be PDHC itself. The other peak comprises the activity due to free component enzyme. Molecular weights of pyruvate dehydrogenase (PDHase), lipoate acetyltransferase (LATase) and lipoamide reductase (LiRase) in free states were estimated at 190,000, 300,000 and 110,000, respectively. Mutations in *ace-4* gene affect LATase not to aggregate to form the core of PDHC. As a result, *ace-4* mutants show high activities of free component enzymes, PDHase and LiRase. Mutants of *ace-2* and *ace-3* genes have the activities of all the three component enzymes of PDHC. Correspondence between *ace-2* and *ace-3* genes and component enzymes of PDHC could not be determined.

INTRODUCTION

Pyruvate dehydrogenase complex (PDHC) has been isolated as a multienzyme complex from *Escherichia coli* (Koike *et al.* 1960, 1963; Eley *et al.* 1972) and mammalian mitochondria (Hayakawa *et al.* 1969; Barrera *et al.* 1972) and studied in full detail. PDHC consists of three enzyme components: pyruvate dehydrogenase (PDHase, EC 1.2.4.1), lipoate acetyltransferase (LATase, EC 2.3.1.12) and lipoamide reductase (LiRase,

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EC 1.6.4.3). Similar PDHC has been isolated from *Saccharomyces cerevisiae* (Wais *et al.* 1973) and *Neurospora crassa* (Harding *et al.* 1970; Wieland *et al.* 1972). The size and structure of the PDHC from *N. crassa* has been studied by electron microscopy and ultracentrifugation (Harding *et al.* 1970). Regulatory kinase and phosphatase subunits are also included in PDHC of *N. crassa* (Wieland *et al.* 1972).

Investigation of PDHC from genetic viewpoint has been reported in *E. coli* (Guest 1974) and in *Salmonella typhimurium* (Langley and Guest 1974). In these bacteria, the structural genes of the three component enzymes are arranged contiguously one another, and seem to form *ace* operon. In the case of eukaryote, two unlinked genes for PDHC have been reported in *Aspergillus nidulans* (Payton *et al.* 1977).

In the preceding paper, we have reported genetic studies of seven acetate genes in *N. crassa* (Kuwana and Okumura 1979). This paper deals with complementation and gene-protein relationships in the acetate mutants deficient in PDHC activity.

MATERIALS AND METHODS

The strains of *Neurospora crassa*, used in the present study have been described in the preceding paper (Kuwana and Okumura 1979). In order to obtain mycelium for enzyme assay, the wild type strain, KGa, was grown at 34°C for one day in Vogel's minimal medium (Vogel 1964), with 2% sucrose or with 2% sucrose plus 0.3% sodium acetate. Culture was aerated with an air pump for tropical fish culture. Acetate mutants were grown for 3 to 5 days with 2% sucrose plus 0.3% acetate. Usually, starvation culture was performed, because PDHC was repressed by acetate and acetate mutants require acetate for growth. The mycelium grown on acetate-supplemented medium was transferred to minimal medium and was incubated again.

Sodium pyruvate, NAD⁺, NADH and ATP were products of Wako Pure Chemical Industries, Ltd., coenzyme A (CoA), acetylphosphate and phosphotransacetylase of Boeringer-Mannheim GmbH, and DL-lipoamide of Sigma Chemical Co. DL-dihydro-lipoamide was synthesized from DL-lipoamide by the method described by Reed *et al.* (1958).

All preparations for enzymes were carried out at near 0°C.

Preparation of PDHC. PDHC was prepared in two ways. Mitochondrial 50,000–220,000×*g* precipitate (M5–22P) was prepared as follows. Mycelium was ground with an equal weight of acid-washed sand and 3 to 5 volumes of grinding buffer, which contained 0.25M sucrose, 0.043mM thiamine pyrophosphate (TPP), 0.4 mM MnSO₄, 5.3 mM L-cysteine and 0.15% bovine serum albumin (BSA) in 0.02M potassium phosphate buffer, pH 7.5. The homogenate was squeezed through gauze, and the filtrate was centrifuged at 1,000×*g* for 15 min. The supernatant was then centrifuged at 10,000×*g* for 20 min. Resulted pellet was washed once with the grinding buffer. Washed mitochondrial pellet was suspended in 0.1M Tris-HCl buffer, pH 7.8, containing 0.1M sucrose. This suspension was sonicated using Tomy model UR-150P sonicator for 20 sec with a setting of 5.5A, and centrifuged at 50,000×*g* for 20 min. The supernatant (M5S) was centrifuged at 220,000×*g*, for 20 min again. The pellet was suspended in 0.02M potassium

phosphate buffer, pH 7.0, containing 2 mM dithiothreitol, 0.5 mM TPP and 0.5 mM MnSO_4 (DTT buffer). This suspension is M5-22P for PDHC assay.

Crude 50,000–220,000 $\times g$ precipitate (C5-22P) was prepared as follows. The first 10,000 $\times g$ supernatant described above was centrifuged at 50,000 $\times g$ for 20 min and this supernatant (C5S) was again centrifuged at 220,000 $\times g$ for 20 min. Resulted pellet was suspended in DTT buffer. This suspension is C5-22P for PDHC assay.

Preparation of component enzymes of PDHC. M5S, M5-22P and M22S fractions were prepared as follows. Mycelium was ground with sand in BSA buffer, which contained 0.25M sucrose and 0.15% BSA in 0.02M potassium phosphate buffer, pH 7.5. From this homogenate, mitochondrial pellet was obtained by the same procedure as described in preparation of PDHC. The pellet was suspended in 0.02M potassium phosphate buffer, pH 7.5, and sonicated as described above. This suspension was centrifuged at 50,000 $\times g$ for 20 min. The supernatant was used as mitochondrial 50,000 $\times g$ supernatant (M5S) for LATase assay. This supernatant was centrifuged at 220,000 $\times g$ for 20 min. The pellet was suspended in 0.02M potassium phosphate buffer, pH 7.5, and was used as mitochondrial 50,000–220,000 $\times g$ pellet (M5-22P). The supernatant was used as mitochondrial 220,000 $\times g$ supernatant (M22S). M5-22P and M22S were used for PDHase and LiRase assays.

Sephadex G-200 gel chromatography. Sephadex G-200 (Pharmacia, Inc.) was soaked in 0.02M potassium phosphate buffer, pH 7.5, for 2 weeks. A 2.6 $\times$ 45 cm column was packed with about 240 ml of the Sephadex and equilibrated with the same buffer. The sample for Sephadex G-200 gel chromatography was prepared by grinding mycelium with sand in 0.02M potassium phosphate buffer, pH 7.5. The homogenate was centrifuged at 50,000 $\times g$ for 20 min. From 2.5 to 5 ml of this supernatant was applied to the Sephadex column. This fraction contained not only PDHC but also three component enzymes of PDHC. Elution was carried out with the same buffer and eluate was fractionated by 4.5 ml. Blue-Dextran 2000 (Pharmacia, Inc.) was used to determine the void volume. The position of a protein in eluate was varied slightly with columns.

Enzyme assay. PDHC activity was measured by monitoring NADH formation spectrophotometrically at 340 nm as described by Harding *et al.* (1970). Specific activity of PDHC was expressed as μ moles NADH per mg protein per hour.

PDHase assay was based on colorimetric determination of ferrocyanide produced by oxidative decarboxylation of pyruvate with ferricyanide as electron acceptor (Reed and Willms 1966), except that 0.5M sodium pyruvate was used for the substrate. Specific activity was expressed as μ moles pyruvate per mg protein per hour.

LATase activity was measured by the method of Reed and Willms (1966). A standard curve was prepared with acetylphosphate (Lipmann and Tuttle 1945), instead of S-acetyldihydrolipoamide. Specific activity was expressed as μ moles S-acetyldihydrolipoamide per mg protein per hour.

LiRase activity was determined by the method of Reed and Willms (1966). The assay was based on spectrophotometric determination of the rate of NADH oxidation in the presence of DL-lipoamide at pH 6.3 (lipoamide-NADH system). The assay based

on reverse reaction was also used in the presence of DL-dihydrolipoamide and NAD^+ at pH 7.5 (dihydrolipoamide- NAD^+ system). Specific activity was expressed as $\mu\text{moles NADH per mg protein per hour}$.

PDHC and LiRase assays were carried out at 30°C , and PDHase and LATase assays at 37°C .

Protein was determined by the method of Lowry *et al.* (1951).

Complementation tests. Complementation in growth was tested on minimal agar plate at the center of which a drop of conidial suspension was inoculated singly or in combination. Surpassing hyphal extension from combined inoculum over that from single inoculum was taken as positive complementation.

In vivo complementation of PDHC activity was determined using heterokaryotic mycelium of two strains. Inoculum cultures of two strains were added together to liquid Vogel's minimal medium and cultivated under aeration at 34°C for 3 to 5 days.

The ability to complement *in vitro* was tested by looking for the appearance of PDHC activity when cell-free extracts of mutants were mixed in combinations. The C5-22P fraction was prepared from the mixture of C5S fractions of two strains. The M5-22P fraction was prepared from the mixture of M5S fractions of two strains. Both the C5-22P and M5-22P fractions were used for the enzyme assay.

RESULTS

Characterization of PDHC. The maximum activity of PDHC was obtained 10 hours after transferring the mycelium from acetate-supplemented medium to minimal medium in both the wild type strain, KGa, and the acetate-requiring strain, 512a (*ace-5*) (Fig. 1).

The optimum pH of PDHC of the wild type was 7.7 using Tris-HCl buffer and potassium phosphate buffer. The K_m values for pyruvate, CoA and NAD^+ were

Fig. 1. Effect of starvation period on PDHC activity. The mycelium grown in Vogel's minimal medium supplemented with acetate was transferred to minimal medium and PDHC was extracted at the indicated time. ○, M5-22P of KGa (wild type); ●, C5-22P of KGa; △, M5-22P of 512a (*ace-5*); ▲, C5-22P of 512a.

Table 1. Activity of PDHC in various strains

Strain (Genotype)	Specific activity (μ mole/mg protein/h)	
	M5-22P	C5-22P
Y2492a (<i>ace-1</i>)	1.1	0.4
511a (<i>ace-2</i>)	0	0
526a (")	0	0.2
536a (<i>ace-3</i>)	0	0
508a (<i>ace-4</i>)	0	0
523a (")	0	0
512a (<i>ace-5</i>)	2.3	2.5
513a (")	0.7	
509a (<i>ace-6=suc</i>)	0.7	0.3
510a (")	0.6	
539a (<i>ace-7</i>)	1.3	
KGa (wild type)	3.2	0.4

Fig. 2. Elution patterns of PDHC activity from Sephadex G-200 column in KGa (wild type) (A) and 508a (*ace-4*) (B).

0.29 mM, 0.013 mM and 0.39 mM, respectively. Magnesium ion (Mg^{2+}) slightly stimulated PDHC activity at a concentration of 1-10 mM, but higher concentration was inhibitory. ATP markedly inhibited PDHC activity. These characteristics are similar to those reported by Harding *et al.* (1970) and Wieland *et al.* (1972) on purified PDHC preparations of *N. crassa*.

The PDHC activity from mitochondrial extract was unstable. The activity was remained only 10% of the initial activity after 7.5 hours at 0°C, while 60% of the activity was remained after 5 days at -20°C. On the other hand, PDHC from crude extract was very stable. The activity was not lost at least 8 days at both 0°C and -20°C.

523	508	526	536	511	
-	+	+	+	+	523
	-	+	+	+	508
		-	+	-	526
			-	+	536
				-	511

Fig. 3. Results of complementation test in growth of heterokaryon made by pairwise combinations of five acetate mutants. 511a and 526a belong to *ace-2*, 536a to *ace-3*, and 508a and 523a to *ace-4*. +, Complementing; -, non-complementing.

Table 2. Restoration of PDHC activity in mixtures of cell-free extracts of PDHC mutants

Combination of strains (Genotype)	Specific activity (μ moles/mg protein/h)	
	M5-22P	C5-22P
511a (<i>ace-2</i>)	0	0
526a (<i>ace-2</i>)	0	0
536a (<i>ace-3</i>)	0	0
508a (<i>ace-4</i>)	0	0
523a (<i>ace-4</i>)	0	0
511a + 526a	0	0
511a + 536a	0	0
526a + 536a	0	0
508a + 523a	0	0
511a + 508a	0.6	1.4
511a + 523a	0.8	0.6
526a + 508a	0.2	0.6
526a + 523a	0.4	1.2
536a + 508a	0.2	0.7
536a + 523a	0.2	0.6
KGa (wild type)	3.2	0.4

PDHC activity. Activity of PDHC in the wild type and acetate mutant strains is shown in Table 1. Mutants of *ace-2*, *ace-3* and *ace-4* genes lack PDHC activity, while the other acetate mutant and the wild type strains definitely showed it.

When extract of the wild type strain, KGa, was subjected to Sephadex G-200 gel chromatography, PDHC activity was obtained at void volume (Fig. 2A), while the activity was not detected in the case of *ace-4* strain, 508a (Fig. 2B). The other four strains that belong to *ace-2* (511a and 526a), *ace-3* (536a) and *ace-4* (523a) showed the same elution pattern as strain 508a.

Complementation test. Nine out of ten pairwise combinations of the five acetate mutants deficient in PDHC activity showed positive complementation in growth on minimal medium (Fig. 3). The combination 508a+523a, both of which belong to *ace-4*, showed intra-genic complementation. In all the nine cases, PDHC activity was detected in the extracts of the mycelium of heterokaryons grown in liquid minimal medium.

Table 2 shows the results of *in vitro* complementation of PDHC activity between acetate mutants. The combination 511a+526a, which did not complement *in vivo*, was also not found to restore PDHC activity *in vitro*. In addition, the combinations 511a+536a, 526a+536a and 508a+523a, which complemented *in vivo*, did not complement *in vitro*.

Fig. 4. Elution patterns of PDHase activity from Sephadex G-200 column in KGa (wild type) (A) and 508a (*ace-4*) (B). ▼, PDHase activity in the absence of ATP; ▽, PDHase activity in the presence of ATP (5mM).

Pyruvate dehydrogenase. PDHase activities of the PDHC-deficient mutant and the wild type strains are shown in Table 3. Every mutant had weak activity compared with the wild type strain, KGa. Characteristically in 508a, PDHase activity in the M22S fraction was higher than that in the M5-22P fraction. This point is clearly shown by Sephadex G-200 gel chromatographic pattern of PDHase activity (Fig. 4). In the wild type strain, KGa, PDHase activity was eluted from the column only at void volume, and this activity was inhibited by ATP (Fig. 4A). Inhibition of PDHase by ATP had been shown in beef kidney by Linn *et al.* (1969). On the contrary, PDHase of the strain 508a (*ace-4*) was eluted at two peaks (Fig. 4B). The first peak was at the void volume like the wild type, but this activity was not inhibited by ATP. The second peak was at fraction 30. The molecular weight of the enzyme at this peak was estimated at about 190,000 from a comparison with Sephadex G-200 chromatographic pattern of standard proteins (Fig. 5). Both 511a (*ace-2*) and 536a (*ace-3*) had weak PDHase activity mainly at void volume.

Lipoate acetyltransferase. LATase activities of PDHC-deficient mutant and the wild type strains are shown in Table 3. The activity in *ace-4* was lower than that in the other strains. Sephadex G-200 gel chromatographic pattern of LATase was investigated. LATase activity of the wild type strain, KGa, was obtained at void volume (peak L) and at fraction 27 (peak S), which had lower activity than peak L (Fig. 6A). The LATase activity of the *ace-4* strain, 508a, was obtained at fraction 27 (peak S) with the same level as the wild type. The peak L, however, was deficient almost perfectly in 508a (Fig. 6B). Owing to the presence of peak S, 508a had low LATase activity (Table 3). The molecular weight of the enzyme at peak S was estimated at about 300,000 (Fig. 5). LATase at peak S of KGa and 508a was compared qualitatively. The K_m value for DL-dihydrolipoamide was calculated to be 29 mM in

Fig. 5. Estimation of the molecular weights of PDHase, LATase and LiRase. Logarithms of the molecular weights of marker proteins were plotted against fraction number after chromatography on Sephadex G-200 column. Fer, Ferritin; Cat, Catalase; Alb, Hen egg albumin; Cyt, Cytochrome C.

Fig. 6. Elution patterns of LATase activity from Sephadex G-200 column in KGa (wild type) (A) and 508a (*ace-4*) (B).

Table 3. Activities of component enzymes of PDHC

Strain (Genotype)	Specific activity (μmoles/mg protein/h)				
	Pyruvate dehydrogenase		Lipoate acetyl-transferase	Lipoamide reductase	
	M5-22P	M22S	M5S	M5-22P	M22S
511a (<i>ace-2</i>)	0.30	0.06	4.3	4.8	7.2
536a (<i>ace-3</i>)	0.20	0.03	8.0	11.4	11.4
508a (<i>ace-4</i>)	0.09	0.18	1.1	5.4	11.4
KGa (wild type)	1.12	0.37	6.1	17.4	18.6

both strains. A broad pH optimum between 8 and 9 was found in both strains. With respect to heat stability, however, enzyme from KGa and 508a were slightly different. The half-lives of the peak S of KGa and that of 508a from starvation cultures were 6 min and 7-8 min, respectively, at 70°C. Specificity of LATase in both peak L and peak S from KGa was also compared. Both enzymes were shown to be completely dependent on dihydrolipoamide, CoA, acetylphosphate and phosphotransacetylase.

Fig. 7. Elution pattern of LiRase activity from Sephadex G-200 column in KGa (wild type).

Fig. 8. Total activity of LiRase eluted from Sephadex G-200 column in various strains. L, total activity of peak L; S, total activity of peak S.

Omission of any element caused no activity. Acetyl residue was transferred to only dihydrolipoamide, and not to 2-mercaptoethanol, dithiothreitol, glutathione and L-cysteine in both peak L and peak S.

Lipoamide reductase. All of the mutants deficient in PDHC activity had LiRase activity (Table 3). Qualitative difference of LiRase between the wild type and mutants was not detected in Km value for substrate, catalytic activity for both directions and heat stability at 70°C. In a Sephadex G-200 gel chromatography, LiRase activity was obtained at void volume (peak L) and at fraction 35 (peak S) in the wild type (Fig. 7). The molecular weight of the peak S enzyme was estimated at about 110,000 (Fig. 5). The total activity of peak L plus peak S was rather uniform in *ace-2*, *ace-3* and the wild type. In contrast to these strains, higher total activity was detected in *ace-4* mutants; the activity of peak S was increased (Fig. 8).

DISCUSSION

Acetate-requiring mutants would be expected to have deficiencies in the carbohydrate metabolism concerned with energy production and the syntheses of precursors for many cellular components. Really, PDHC-deficient strains have been found in acetate-requiring mutants in *Escherichia coli* (Henning *et al.* 1964; Guest 1974), *Salmonella typhimurium* (Langley and Guest 1974) and *Aspergillus nidulans* (Payton *et al.* 1977). We identified three classes of mutants as deficient in PDHC activity among acetate mutants of *Neurospora crassa* (Table 1).

Characteristics of PDHC in crude *Neurospora* extract were similar in many respects to the purified enzyme by Harding *et al.* (1970) and Wieland *et al.* (1972). Repression of PDHC by acetate was common property both in *N. crassa* (Fig. 1) and in *E. coli* (Dietrich and Henning 1970), while PDHC of *A. nidulans* was not repressed by acetate (Payton *et al.* 1977).

All the heterokaryons, which showed positive complementation in growth on minimal medium (Fig. 3), showed significant levels of PDHC activity. The combination 508a+523a showed intra-genic complementation, and would be expected to form hybrid enzyme. The PDHC activity was recovered *in vitro* by mixing cell-free extracts in six combinations among nine complemented *in vivo* (Table 2). The component enzymes would readily be aggregated to form active PDHC in these combinations.

Each of the three component enzymes of PDHC was detected as two peaks by Sephadex G-200 gel chromatography. One of them eluted from Sephadex column at void volume comprises high molecular weight aggregate. In the case of the wild type strain, it may be PDHC itself. The other peak comprises the activity due to free component enzyme. Molecular weights of the three component enzymes of PDHC, PDHase, LATase and LiRase were about 190,000, 300,000 and 110,000, respectively (Fig. 5). These values are similar to those of *E. coli* enzymes except for LATase (Eley *et al.* 1972).

The ferricyanide assay is rather nonspecific method for PDHase, since other enzymes also catalyze oxidative decarboxylation of pyruvate. And yet, Payton *et al.* (1977) failed to assay PDHase activity using ferricyanide as an electron acceptor. Using Sephadex G-200 gel chromatography, however, we detected two PDHase peaks by this method. Presumably, one is attributable to PDHC and the other is free component enzyme (Fig. 4).

The activities of all component enzymes were detected in *ace-2* and *ace-3* mutants (Table 3), and we were unable to conclude which components were affected in these two mutant classes. The products of the genes *ace-2* and *ace-3* might be some proteins existing in PDHC and have some regulatory roles. In fact, Wieland *et al.* (1972) reported that *Neurospora* PDHC contains regulatory kinase and phosphatase subunits.

It is sure that the mutations in *ace-4* gene affect LATase, although *ace-4* mutants had low level of LATase activity owing to the presence of peak S (Table 3, Fig. 6). There may be three possibilities for the existence of peak S. The first possibility is that *Neurospora* has two LATase isozymes and peak S is one of them, which is not the component of PDHC. Really, there are at least three thioltransacetylase isozymes

in *Clostridium kluyveri* and in pigeon liver, and two of them can transfer the acetyl residue of acetyl-CoA to 2-mercaptoethanol besides dihydrolipoamide (Brady and Stadtman 1954). In our case, however, peak S had strict substrate specificity and did not catalyze the reaction transferring acetyl residue to 2-mercaptoethanol. So, we did not consider that peak S is unrelated activity of LATase component of PDHC.

The second possibility is that *ace-4* is the structural gene for LATase. Then, *ace-4* mutant has LATase activity but the protein changed qualitatively, so that it does not aggregate to molecular weight of more than 300,000. It has been shown in various organisms that LATase consists the core of PDHC (Ishikawa *et al.* 1966; Hayakawa *et al.* 1969; Reed and Cox 1970). Since the core of PDHC is not formed, the free components, PDHase and LiRase, were detected large quantities only in *ace-4* mutants (Fig. 4, Fig. 8). Although Km value and pH optimum were not different between peak S of the wild type and that of *ace-4*, there was a little difference in heat stability. This seems to support the second possibility.

The third possibility is that the *ace-4* gene is the structural gene for some protein which makes LATase subunits aggregate to form the core of PDHC, and LATase *per se* is normal in *ace-4* mutants. There are not too much differences between peak S of the wild type and that of *ace-4* mutants. This could be taken to suggest that *ace-4* is not the structural gene for LATase.

Although neither the second nor the third possibility is decisive at this stage, it seems to be sure that the mutations in *ace-4* gene affected LATase not to aggregate as the core of PDHC.

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