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Properties of Yeast Pyruvate Decarboxylase and their Modification by Proteolytic Enzymes

II. Selective Alteration by Yeast Proteases[1]

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Pyruvate decarboxylase has been shown to be more heat labile in extracts of a mutant strain than in extracts of the parent yeast strain. Mutant extracts were found to contain higher levels of proteolytic enzymes than wild-type extracts, although aging of the latter preparations resulted in the activation of inactive proteases. A partially purified protease preparation was shown to degrade pyruvate decarboxylase in a selective manner such that the ability to form free acetaldehyde was destroyed while the acetylmethylcarbinol-forming activity was increased 60%. Pyruvate, or a mixture of pyruvate and acetaldehyde, was partly successful in protecting pyruvate decarboxylase from the action of the protease. Stabilization of pyruvate decarboxylase by high concentrations of phosphate, sulfate, or glycerol has been shown to be due to the inhibitory action of these compounds on yeast proteases. Pyruvate decarboxylase which was partially degraded by the protease had different relative activities on a series of α-keto acids from the corresponding activities of a nondegraded decarboxylase preparation. The data presented are in accord with a two-site mechanism postulated for pyruvate decarboxylase.

In the previous paper (2) it was demonstrated that yeast pyruvate decarboxylase (EC 4.1.1.1) could be protected against heat denaturation in the presence of cofactors (TPP[2] and Mg^{++}) and a high concentration of phosphate or sulfate ions. Pyruvate decarboxylase from a mutant strain was shown to be more unstable than wild-type enzyme. The mutant enzyme was found to have a lower activity ratio (ratio of the maximum rate of acetaldehyde formation to the maximum rate of AMC[2] synthesis) than that observed for wild-type enzyme, and the activity ratio of the mutant strain was shown to fall upon aging at 5°. According to the two-site mechanism for enzymatic pyruvate decarboxylation (3) it should be possible to find mutant decarboxylases with alterations in second site activity resulting in lower activity ratios. The fact that the activity ratio of the mutant decarboxylase decreased upon aging, however, suggested that some other factor might be operating in crude extracts to account for this phenomenon. The experiments in the present paper have shown that the change in the activity ratio of the mutant decarboxylase is the result of the selective action of a proteolytic enzyme normally found in yeast extracts. The properties of some of the proteases of yeast are described as well as the differences between wild-type and mutant decarboxylase preparations.

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[2] TPP and AMC are abbreviations for thiamine pyrophosphate and acetylmethylcarbinol (acetoin), respectively.

EXPERIMENTAL PROCEDURE

Determination of protease activities. Protease A (nomenclature of Lenney (4)) was determined by a modification of the procedure of Lenney (4). To

2 ml of 2% hemoglobin dissolved in 0.07 N citric acid, and temperature equilibrated at 37°, was added 0.1 ml of a suitable dilution of enzyme preparation. After incubation at 37° for 30 minutes, the reaction was terminated by addition of 1.0 ml of 12% trichloracetic acid and 0.9 ml of water. The precipitated protein was removed by centrifugation and the supernatant fluid was suction filtered through a small diameter disc of filter paper to remove any particles that did not remain well packed when pouring off the supernatant fluid. An enzyme control was also prepared where enzyme was added after acidification of the hemoglobin with trichloroacetic acid. The deproteinized solution was placed in a 1.0-cm spectrophotometer cuvette and the absorbance at 280 mμ was determined. The net absorbance, obtained after subtraction of the absorbance of the enzyme control, was used as a measure of protease A activity.

Protease B was determined by its ability to liberate dye which is conjugated to insoluble particles of denatured collagen (azocoll hide powder) (5). To a 16-mm test tube containing a small teflon-coated magnetic stirring bar was added 20 mg of azocoll, 0.3 ml of 0.1 M potassium phosphate, pH 7.7, and 0.1 ml of 1% Triton X-100. This mixture was temperature equilibrated at 37° and the reaction was initiated by addition of 0.1 ml of a suitable dilution of the enzyme preparation. The tube was placed in a 37° water bath which contained a submerged magnetic stirrer. A control tube with all components added, except for enzyme, was also included. After incubation with stirring for 30 minutes, 3.5 ml of ice-cold water was added to each tube and the contents stirred briefly on an orbital mixer. Colored solutions (pink) were suction filtered through a small diameter disc of filter paper and absorbancies were determined in 1.0-cm cuvettes at 520 mμ. The small absorbance of the control was subtracted from that of the experimental sample. Net absorbancies as high as 0.5 were shown to be a linear function of enzyme concentration or time of incubation with a fixed amount of enzyme, and were used as a measured of protease B activity. Protease A does not liberate dye from azocoll at any pH from 3.0 to 9.0.

Protease C (nomenclature of Hata et al. (6)) was assayed by incubating 0.05 ml of enzyme with 0.15 ml of a solution of 0.01 M *N*-Cbz-L-alanyl-L-phenylalanine in 0.1 M sodium citrate buffer, pH 5.75 at 37°. After 30 minutes 3.8 ml of ninhydrin reagent (7) were added and the amount of L-phenylalanine determined using a known amino acid control tube (0.1 to 0.4 μmoles per tube). The experimental value was subtracted from an enzyme control (no substrate) to correct for free amino groups present in the enzyme preparation before incubation. Lenney and Dalbec (8) have recently shown that highly purified protease B does not hydrolyze various carbobenzoxy-dipeptides including *N*-Cbz-L-alanyl-L-phenylalanine.[3] Félix and Brouillet (9) have described a metal-requiring carboxypeptidase from brewer's yeast which also hydrolyzes *N*-Cbz-L-alanyl-L-phenylalanine, but is completely inhibited by 10^{-3} M EDTA. The activity on this dipeptide substrate that we have observed in our yeast extracts was not inhibited by 10^{-3} M EDTA.

Both protease B and protease C, assayed as described above, were completely inhibited by phenylmethylsulfonyl fluoride (saturated), an inhibitor with properties similar to those of diisopropylphosphofluoridate (10). This finding is consistent with the observation that protease B (4, 6) and protease C (6), but not protease A, are inhibited by diisopropylphosphofluoridate, whereas the carboxypeptidase from yeast is not sensitive to this compound (9).

Other methods and assays. The previous paper (2) describes the assays used for the determination of acetaldehyde, AMC, CO_2, protein, and the activity ratio, as well as methods for growing yeast and preparing cell-free extracts. Sonic extracts of yeast were made by rapid suspension of air-dried cells in 5 volumes of cold (0–5°) 0.1 M sodium phosphate, pH 7.0, and subjecting the suspension to sonic vibration for 30 minutes with a Raytheon 10-Kc oscillator. Yeast proteolytic enzymes were prepared by the method of Lenney (4).

Chemicals. Sodium salts of α-keto acids were prepared as previously described (3). TPP, hemoglobin (bovine), and phenylmethylsulfonyl fluoride were obtained from Sigma Chemical Co. Azocoll was obtained from Calbiochem. *N*-Cbz-L-alanyl-L-phenylalanine was obtained from Cyclo Chemical Corp.

RESULTS

Effect of heating and aging on mutant enzyme. Mutant enzyme, unlike wild-type enzyme, was not very well protected when heated in 0.5 M phosphate buffer in the presence of cofactors (Fig. 1). Although mutant enzyme preparations were always less stable to heating than wild-type enzyme, different preparations displayed varying degrees of heat sensitivity. In spite of the marked destruction of mutant enzyme during heating (Fig. 1), the activity ratio was not changed at the end of the heating period (Table I).

The fact that incubation in phosphate buffers of high concentration, in the pres-

[3] J. F. Lenney, personal communication.

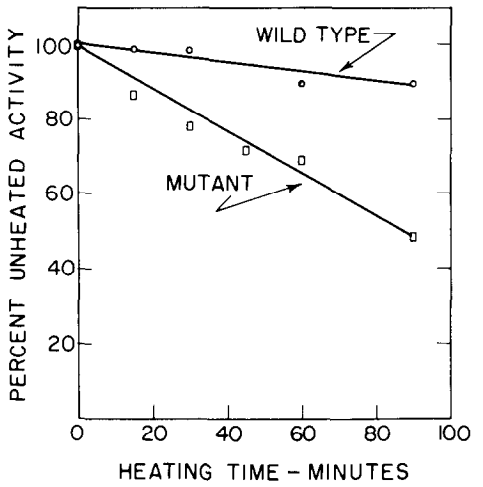

FIG. 1. Residual decarboxylating activity after heating in 0.5 M sodium phosphate buffer as a function of time of heating. All samples contained 0.5 M sodium phosphate buffer, pH 6.3, 2.1×10^{-3} M TPP, 0.002 M $MgSO_4$, and 0.2 ml of respective crude extract per ml of heating mixture. After heating at 60° for the indicated time, the mixtures were cooled in an ice bath and suitable dilutions were assayed manometrically without removing heat denatured precipitated protein.

TABLE I

STABILITY OF WILD-TYPE AND MUTANT ENZYMES TO HEATING IN THE PRESENCE OF COFACTORS

Enzyme[a]	Treatment	Acetaldehyde[b]	AMC[b]	Activity[c] ratio
Wild-type	None	114	2.16	52.7
	Heated[d]	114	2.02	56.4
Mutant	None	10.4	0.44	23.6
	Heated[d]	5.5	0.22	25.0

[a] The extracts were prepared by incubating 1 g of dried cells with 5 ml of 0.1 M sodium phosphate, pH 7.0, for 24 hours at 5°. The cells were removed by centrifugation in the cold.

[b] Values are in μmoles/0.1 ml extract/30 minutes.

[c] Activity ratio = rate of acetaldehyde formation/rate of AMC synthesis.

[d] Heating mixtures contained 0.5 M sodium phosphate, pH 6.3, 1.3×10^{-3} M TPP, 0.002 M $MgSO_4$, and enzyme. Samples were heated at 60° for 30 minutes.

ence of cofactors, conferred heat stability on mutant as well as wild-type enzyme (2), prompted a study of the aging of mutant enzyme in 1.0 M phosphate buffer. Prolonged incubation at 5° of mutant enzyme in this high buffer concentration resulted in a gradual loss of acetaldehyde-forming activity (Fig. 2). By contrast, the rate of AMC formation actually increased 11% in 50 hours of incubation, followed by a gradual loss in activity (Fig. 2). Although this increase was small, it appeared to be characteristic for such preparations and has been observed several times. Aging of mutant enzyme at 5° in 0.1 M phosphate buffer resulted in a gradual loss of both activities (Fig. 2); the rate of acetaldehyde formation decreased more rapidly than the rate of AMC formation.

Alteration of heat sensitivity of wild-type enzyme. Since wild-type enzyme was found to be fairly stable in the cold (2), it was customary to store a batch of new enzyme at −20° and thaw this sample for use as required. It was noted that an "old" preparation that had been frozen and thawed repeatedly over a period of about 3 months was no longer as stable to heating as it had been when it was freshly prepared. A comparison was therefore made of the stability to heating of this "old" preparation and a "new" preparation of the same batch of enzyme which had been kept frozen since it was originally made. Fig. 3 shows that there was indeed a difference in the stability to heating between these two preparations. Although both samples were almost equally stable when heated in 1.0 M phosphate buffer, the "old" preparation was significantly more heat sensitive than the "new" one at lower phosphate concentrations.

Analysis of the activity ratio of the "old" enzyme preparation revealed that it had dropped from a value of 56 to 10.4. It then became evident that wild-type and mutant enzyme did not differ fundamentally, except possibly in the time required for significant decrease of the activity ratio.

Evidence for the action of yeast proteolytic enzymes on pyruvate decarboxylase. Since, in general, dilute enzyme preparations are frequently more readily

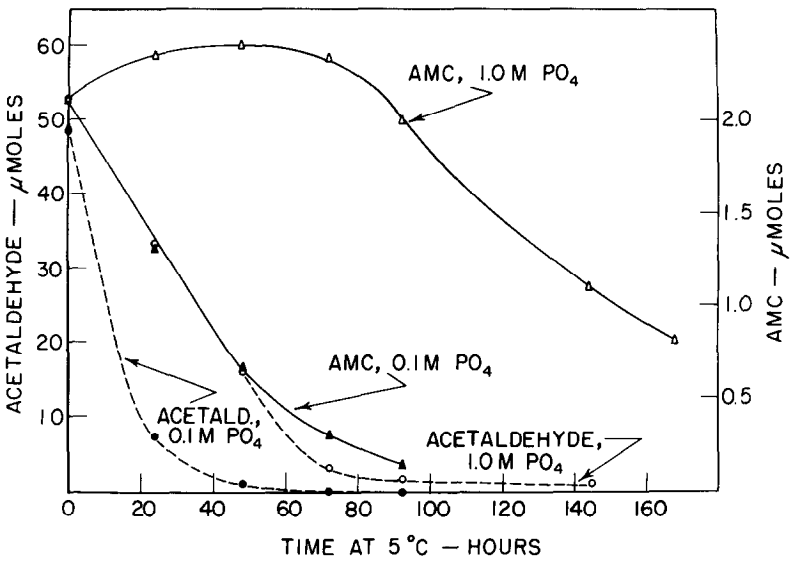

FIG. 2. Recovery of activities of mutant enzyme after aging at 5° in 0.1 M and 1.0 M sodium phosphate buffers as a function of time of aging. Crude mutant extract was concentrated by precipitation with 80% saturated $(NH_4)_2SO_4$ and dissolved in either one-tenth volume of 0.1 M sodium phosphate, pH 7.0 or 1.0 M sodium phosphate, pH 6.3. Both solutions were incubated at 5° and samples were removed at the indicated intervals, and assayed for acetaldehyde and AMC formation. The values given are those calculated for 0.1 ml of incubation mixture assayed for 30 minutes. The actual amount of enzyme used in each assay was suitable for the particular procedure employed (2).

denatured than are more concentrated protein solutions, wild-type decarboxylase was aged at various dilutions in 0.1 M phosphate buffer, pH 6.1, at room temperature, to determine whether working with concentrated solutions would result in greater enzyme stability. After incubation for 20 hours, assays were made of the same amount of enzyme from each of the various dilutions. The results (Table II) yielded the unanticipated finding that the more dilute enzyme solutions were the most stable to aging. It seemed likely that dilution served to reduce the concentration of some destructive agent, such as a proteolytic enzyme, and thus lessen its activity.

Since mutant enzyme was the most unstable preparation, it appeared to be a reasonable starting material for the search for proteolytic activity. Mutant enzyme was therefore concentrated with ammonium sulfate (saturated) and incubated in 0.05 M phosphate buffer, pH 6.1, at 30° for 24 hours. During this period, a copious precipitate of denatured protein appeared which disappeared upon further incubation. The resulting solution, which no longer contained pyruvate-decarboxylating or AMC-synthesizing activities, was tested for its ability to degrade purified wild-type pyruvate decarboxylase. Fig. 4 shows the results of an experiment where the presumed proteolytic preparation from mutant extract was incubated with purified wild-type pyruvate decarboxylase. When incubation took place for 10 minutes in the absence of substrate, the resultant mixture was completely devoid of decarboxylating activity. Although the addition of pyruvate together with proteolytic enzyme served to protect the decarboxylase, the latter enzyme was, nevertheless, progressively inactivated. The control (Fig. 4), without proteolytic preparation (— enzyme), showed the characteristic fall-off in rate due to increasing inhibition by accumulating acetaldehyde (3).

Although acetaldehyde alone did not

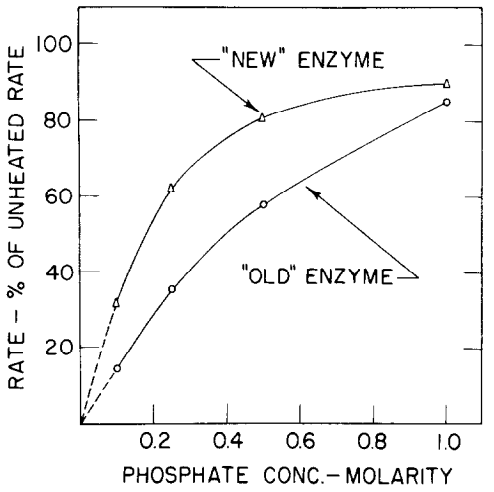

FIG. 3. Residual decarboxylating activity after heating in the presence of cofactors as a function of sodium phosphate concentration for enzyme that had been repeatedly frozen and thawed ("old" enzyme) and the same enzyme preparation that had been frozen and thawed only once ("new" enzyme). All samples were heated at 60° for 30 minutes in sodium phosphate buffers, pH 6.0, of the indicated molarities containing 2.1×10^{-3} M TPP, 0.002 M $MgSO_4$, and 0.1 ml of the respective wild-type extract per ml of heating mixture. Suitable dilutions of the heated mixture were assayed manometrically without removing heat denatured precipitated protein.

TABLE II

EFFECT OF AGING OF VARIOUS DILUTIONS OF WILD-TYPE EXTRACT ON ENZYME INACTIVATION

Dilution of enzyme during aging[a]	Enzyme activity[b] (%)
Undiluted	13.7
1:2	20.8
1:5	27.4
1:10	51.0
1:20	67.7
1:50	94.3

[a] Wild-type enzyme was diluted in 0.1 M sodium phosphate, pH 6.1, and aged at room temperature (approximately 23°) for 20 hours.

[b] Values are given as percent of original unaged pyruvate decarboxylating activity after aging.

serve to protect the decarboxylase from the action of proteolytic enzyme, the combination of pyruvate plue acetaldehyde was more protective than pyruvate alone. This is shown in Fig. 5 where inactivation only became evident after about 100 minutes.

Since pyruvate decarboxylase from mutant cells was degraded selectively upon aging (2), (Fig. 2), the possibility of finding a similar action of the proteolytic enzyme on purified pyruvate decarboxylase from wild-type cells was investigated. Fig. 6 illustrates the selective action of the proteolytic preparation. At the end of a one hour incubation period, at 30°, the rate of acetaldehyde formation was reduced 90%. The rate of AMC synthesis was actually found to increase 60%, this increase being qualitatively similar to the effect observed upon aging of crude mutant enzyme (Fig. 2).

Preliminary studies of proteolytic enzymes from yeast. The most extensive study of the proteolytic enzymes of yeast, prior to the completion of the present work, is that of Lenney (4), who showed that all strains of yeast examined contain

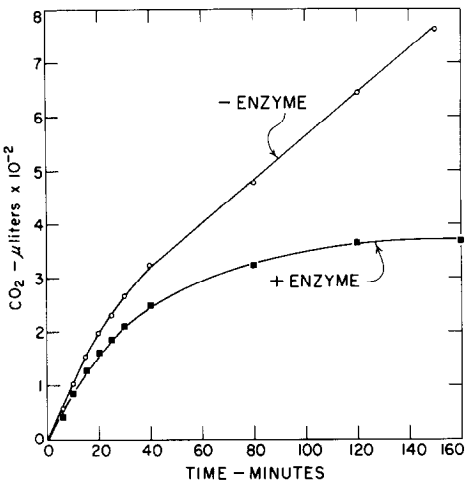

FIG. 4. Action of proteolytic enzymes on pyruvate decarboxylase in the presence of pyruvate. Each Warburg vessel contained 0.125 M sodium phosphate, pH 6.3, 1.3×10^{-3} M TPP, 1.25×10^{-3} M $MgSO_4$, 6.25×10^{-3} M EDTA, and 0.125 M sodium pyruvate. The side arm contained a suitable dilution of the most purified pyruvate decarobxylase preparation (2). Upon addition of decarboxylase, the total volume was 1.6 ml. Experimental vessels contained in addition 0.4 ml of a proteolytic enzyme preparation (+ enzyme) whereas control vessels lacked proteolytic enzyme (− enzyme). The vessels were shaken at 30°. The atmosphere was air.

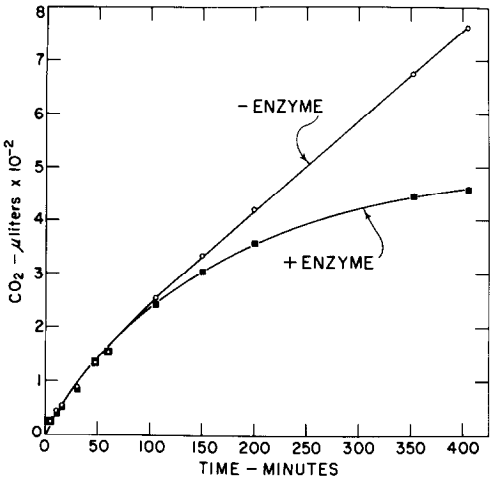

FIG. 5. Action of proteolytic enzymes on pyruvate decarboxylase in the presence of pyruvate and acetaldehyde. Each Warburg vessel contained 0.125 M sodium phosphate, pH 6.3, 1.3 × 10^{-3} M TPP, 1.25 × 10^{-3} M $MgSO_4$, 6.25 × 10^{-3} M EDTA, 0.125 M sodium pyruvate, and 0.125 M acetaldehyde. The side arm contained a suitable dilution of the most purified pyruvate decarboxylase preparation (2). Upon addition of decarboxylase the total volume was 1.6 ml. Experimental vessels contained, in addition, 0.4 ml of a proteolytic enzyme preparation (+ enzyme) whereas control vessels lacked proteolytic enzyme (− enzyme). The vessels were shaken at 30°. The atmosphere was air.

two distinct proteolytic enzymes. Protease A (Lenney's terminology) is a pepsin-like enzyme with an acidic pH optimum, whereas protease B is most active near neutrality. We have confirmed and extended Lenney's findings. The details of our protease studies will be presented elsewhere, but a few of our results will be mentioned here. As Lenney showed (4), both proteases must be activated when they are first obtained in crude extracts before they can degrade suitable assay substrates. We have demonstrated that protease A is apparently responsible for activation of itself as well as protease B. Preliminary results, making use of a new procedure for the separation of the proteases, indicate that protease A degrades pyruvate decarboxylase without altering its activity ratio. By contrast, protease B appears to degrade pyruvate decarboxylase selectively in such a manner that it loses its ability to split 2-hydroxyethyl TPP but still retains its activity in forming this complex; the synthesis of AMC being used as an indicator of the formation of the TPP complex.

Enzyme stabilization by inhibition of proteolytic activity. Since the proteases in yeast contribute to the instability of pyruvate decarboxylase, it seemed likely that the decarboxylase could be stabilized by addition of inhibitors of the proteases. The fact that high concentrations of ammonium sulfate (11) and glycerol (12) have been reported to stabilize pyruvate decarboxylase preparations suggested that these materials act by inhibiting proteolysis. It was shown that incubation of a crude yeast decarboxylase preparation in 1.3 M ammonium sulfate or 50% glycerol at 30° for 14 hours did not give rise to the precipitation of denatured protein which is characteristic for extracts prepared and incubated in water

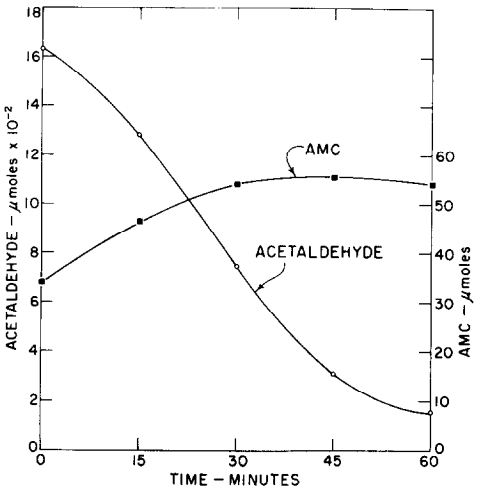

FIG. 6. Selective degradation of pyruvate decarboxylase by proteolytic enzymes as a function of time. Incubation mixtures contained 0.1 ml of the most purified pyruvate decarboxylase preparation (2), 0.2 ml of a proteolytic enzyme preparation, and 0.2 M sodium phosphate, pH 6.3 in a total volume of 0.5 ml. Proteolytic enzyme was not added to the zero time sample. After incubation at 30° for the indicated time, ice-cold 1.0 M sodium phosphate buffer, pH 6.3, was added to give a suitable dilution for assay for acetaldehyde and AMC.

or in 0.1 M phosphate buffer. Also, pyruvate decarboxylase survived aging significantly better in 1.0 M phosphate buffer, 50% glycerol, and 1.3 M ammonium sulfate solutions than in 0.1 M phosphate buffer.

The possibility that the protection against heating conferred by high concentrations of phosphate and ammonium sulfate might, in fact, be due completely to an inhibitory action of these anions on proteolytic activity was tested by substituting glycerol for phosphate or ammonium sulfate in heating experiments. When pyruvate decarboxylase preparations were heated for 30 minutes at 60° in the presence of 40–50% glycerol, TPP, and Mg^{++} ions, complete recovery of decarboxylating activity was achieved. When tested with an enzyme preparation resolved for cofactors, glycerol was only effective in protecting against heat denaturation when the cofactors were also present; a situation similar to that obtained with 1.0 M phosphate (2).

High concentrations of phosphate, sulfate, and glycerol were effective in inhibiting protease B (Table III). High concentrations of phosphate and sulfate could not be used to test for possible inhibition of protease A since the concentrations of these compounds tested precipitated the hemoglobin used as substrate in the assay for this enzyme. Inhibition of protease A by 50% glycerol was relatively weak, however (Table III).

The most highly purified preparations of pyruvate decarobxylase obtained to date in this laboratory have been found to contain protease A activity and the inactive precursor of enzyme B. These proteases were also shown to be present in the final product obtained using the purification procedure of Holzer and Beaucamp (13). Unfortunately, the heating step used in our purification procedure (2), although denaturing protease B, does not completely destroy protease A or the inactive precursor of protease B.

Preliminary results with a yeast pyruvate decarboxylase essentially free of protease activity revealed that the presence of TPP and Mg^{++} alone was sufficient to protect the decarboxylase against heat denaturation in the absence of high concentrations of phosphate buffer. The requirements for stabilization to heating therefore appear to be similar to those found for pyruvate decarboxylases from *Acetobacter suboxydans* and *Zymomonas mobilis* (2).

Differences between mutant and wild-type extracts. During the usual extraction of dried cells at 5° the pH dropped from 7.0 to about 6.4. Wild-type extracts (24 hour extractions) were found to contain low levels of protease A activity and no detectable protease B (Table IV). By contrast, mutant extracts (24 hour extraction) were found to contain high levels of protease A and low but measurable levels of protease B. Further incubation of both extracts at 5° or at room temperature resulted in increased activation of protease B in mutant extracts, but no corresponding increase in the wild-type extracts (Table IV). Upon adjustment of extracts to pH 5.0, the pH that Lenney had shown (4) to be optimum for protease activation, protease B levels increased but always remained lower for wild-type extracts than for mutant ex-

TABLE III

INHIBITION OF YEAST PROTEASES BY COMPOUNDS THAT STABILIZE PYRUVATE DECARBOXYLASE

Inhibitor	Inhibition[a]		
	Protease A[b]	Protease B[b]	Protease C[b, c]
0.5 M sodium phosphate	—	29	24.7
1.0 M sodium phosphate	—	91.5	33.0
0.7 M ammonium sulfate	—	80	—
50% glycerol	27.8	100	39.5

[a] Values are percent inhibiton compared with protease activity in the absence of inhibitor.

[b] Protease activity determined as described in "Experimental Procedure."

[c] These assays were performed after the report of the occurrence of protease C in yeast extracts (6, 8).

TABLE IV

ACTIVATION OF PROTEASES IN CRUDE EXTRACTS OF WILD-TYPE AND MUTANT CELLS

The unadjusted pH values of the extracts were pH 6.6 for the wild-type extract and pH 6.3 for the mutant extract. The extracts were prepared by incubating 1 g of dried cells with 5 ml of 0.1 M sodium phosphate, pH 7.0, for 24 hours at 5°.

Treatment	Wild-type cells		Mutant cells	
	Protease A[a]	Protease B[b]	Protease A[a]	Protease B[b]
Extraction for 24 hours at 5°	0.060	0.004	0.510	0.138
Extraction for 24 hours at 5° plus incubation of extract at room temperature for 16 hours	0.050	0.016	0.424	0.625
Extraction for 24 hours at 5°, acidification to pH 5.0, and incubation at 30° for 16 hours	0.170	0.200	0.474	1.830
Extraction for 24 hours at 5°, acidification to pH 5.0, and incubation at 30° for 36 hours	—	0.260	—	1.530

[a] The values for protease A are the readings obtained using 0.1 ml of extract and acid-hemoglobin substrate in the assay described in "Experimental Procedure."

[b] The values for protease B are the readings obtained using 0.1 ml of extract and azocoll substrate in the assay described in "Experimental Procedure."

tracts (Table IV). It would thus appear that the proteases in extracts of the mutant strain are more readily activated and rise to higher levels than those in extracts of the wild-type strain. This may reflect differences in the nature of the proteins in the respective extracts since activation appears to represent, at least in part, digestion of protein which can compete with externally supplied substrates for the active sites of the proteases. When a fully activated protease was added to a crude unactivated wild-type extract, the protease B activity of the added preparation was almost completely masked by the protein in the crude extract. It is also of interest to note that whereas the final cell yield of mutant and wild-type strains is the same, the mutant strain has been shown to have a significantly lower growth rate. The exact alteration in the mutant strain is not known.

After this work was completed a report appeared describing the presence of yet a third protease (protease C) in extracts of yeast (6). Subsequent examination of our extracts confirmed the presence of protease C. It has been shown recently that activation of protease C parallels the activation of protease B (14). In view of the discovery of a third protease in yeast extracts it was necessary to determine whether the azocoll assay (see Experimental Procedure) used to determine protease B was actually measuring protease B and not protease C activity. It was shown that the azocoll assay is indeed specific for protease B by following the relative disappearance of azocoll activity and protease C activity as a function of the time of aging of a dilute extract at room temperature. It was observed that protease C activity was fairly stable while azocoll activity gradually disappeared after several days of incubation. It has been demonstrated (6) that protease B is less stable than protease C. It is not yet known which protease is responsible for the selective degradation of pyruvate decarboxylase (Fig. 6) and our present efforts seek to clarify this point. The fact that protease C is not strongly inhibited by 1.0 M sodium phosphate or 50% glycerol (Table III) suggests that this enzyme may not be responsible for the selective degradation of pyruvate decarboxylase since high concentrations of phosphate or glycerol serve to protect the decarboxylase.

Action of mutant and wild-type enzymes on various α-keto acids. The action of mutant and wild-type enzymes was examined using several α-keto acids as substrates (Table V). The mutant enzyme

TABLE V

DECARBOXYLATION OF VARIOUS α-KETO ACIDS BY WILD-TYPE AND MUTANT ENZYMES

The values are initial rates of decarboxylation of the various α-keto acids relative to the rate of decarboxylation of pyruvate. All assays were performed manometrically. Reaction mixtures contained 0.125 M sodium phosphate, pH 6.3, 1.3×10^{-3} M TPP, 1.25×10^{-3} M $MgSO_4$, and 0.125 M sodium salt of the respective α-keto acid. Total volume, 1.6 ml. Atmosphere, air. Vessels were shaken at 30°.

Enzyme	Pyruvate	α-keto-butyrate	α-keto-valerate	α-ketoiso-valerate	Activity ratio[c]
Wild-type	100	76	40	25	57.4
Mutant[a]	100	73	55	31	55.5
Mutant[b]	100	402	146	308	1.7

[a] This was a sonic extract assayed immediately after preparation.

[b] This was an extract of dried cells which was aged at 5° to give a low activity ratio (2).

[c] Activity ratio = rate of acetaldehyde formation/rate of AMC synthesis.

which had an activity ratio of 55.5, essentially that for wild-type enzyme, was obtained by rapid sonic disruption rather than through the slow extraction procedure previously used (2). This mutant preparation closely resembled wild-type enzyme in its ability to decarboxylate the different α-keto acids used. Mutant enzyme, which was aged until it had an activity ratio of 1.7, was significantly more active with α-ketobutyrate, α-ketovalerate, and α-ketoisovalerate than with pyruvate.

DISCUSSION

Previous studies on the mechanism of action of pyruvate decarboxylase (3) led to the proposal that this enzyme contains two distinct catalytic sites; the first site being concerned with the condensation of pyruvate with enzyme-bound TPP, while the second site acts to liberate free acetaldehyde from the 2-hydroxyethyl TPP intermediate of pyruvate decarboxylation. In the light of this hypothesis it seemed reasonable to expect to be able to find mutant yeast strains having altered pyruvate decarboxylase proteins. One such mutant enzyme could conceivably retain the ability to form enzyme-bound 2-hydroxyethyl TPP (normal site 1) but be unable to liberate free acetaldehyde from this complex (defective site 2). It should be possible, however, for such a mutant enzyme to synthesize AMC from pyruvate and acetaldehyde since 2-hydroxyethyl TPP, the intermediate for condensation with acetaldehyde, would be formed.

The data presented above appear to confirm the original proposal. Although our efforts to find a mutant having an altered decarboxylase were not successful, we have, nevertheless, been able to demonstrate the feasibility of selectively destroying the second catalytic site of pyruvate decarboxylase. The mutant strain isolated differs from the wild-type yeast in that proteolytic enzymes, also present in this organism, are more readily activated and rise to higher levels in extracts of the former strain. Once activated, protease B (optimal proteolytic activity near neutrality) can alter the activity ratio (ratio of the rate of acetaldehyde formation to the rate of AMC synthesis) of partially purified wild-type pyruvate decarboxylase (Fig. 6) by apparent destruction of the second catalytic site of the decarboxylase. The fact that the maximum rate of AMC synthesis increased 60% during second site destruction (Fig. 6) may be a consequence of decreasing the competition for enzyme-bound 2-hydroxyethyl TPP. When no longer catalytically dissociated to free acetaldehyde, more of this complex may be available for condensation with added acetaldehyde to form AMC at an increased rate.

Since high concentrations of sodium phosphate, ammonium sulfate (also sodium sulfate) (2) or glycerol serve for stabilization of the decarboxylase to heat denaturation, and also act as inhibitors of protease B (Table III), it seems fairly certain that the stabilizing effects of these agents for many enzymes from yeast may have the same common basis.

The fact that mutant extracts, unlike

wild-type extracts, can readily activate protease B accounts for the rapid fall in activity ratio of the former preparations (2). Since heating at 60° most readily destroys protease B, it was not surprising to find that prolonged heating of a mutant extract did not result in a significant change in activity ratio (Table I). The marked drop in decarboxylating activity of the mutant extract during heating at 60° (Fig. 1) is explained in terms of the higher level of relatively heat stable protease A (acidic pH optimum) present in the mutant preparation. Since protease A does not degrade pyruvate decarboxylase selectively, it can destroy the decarboxylase without altering the activity ratio (Table I). Although high concentrations of inorganic phosphate strongly inhibit protease B (Table III), inhibition does not appear to be complete and selective proteolysis can continue slowly even at 5° (Fig. 2). When enzyme from mutant cells was obtained by rapid sonic disruption, rather than by the slow extraction procedure usually used, it was shown that this preparation had an activity ratio as high as that of wild-type enzymes (Table V). This result demonstrates that the proteases are not active in intact cells and that mutant decarboxylase is most probably identical in every way with wild-type enzyme. Preliminary results of experiments now in progress show that the levels of proteases that are found in crude extracts depend upon the particular strain of yeast used as well as the phase of growth of the harvested cells.

Matile and Wiemken (15) have demonstrated recently that the proteases and other hydrolytic enzymes of yeast occur in the vacuole, which they consider to be functionally equivalent to the lysosomes of mammalian cells. Matile (16) has also shown that proteolytic enzymes of *Neurospora crassa* are contained in intracellular spherical particles which are enclosed by a single membrane envolope (17). There appear to be at least two proteases in Neurospora (16) similar to proteases A and B described by Lenney (4) in yeast.

The protease levels in crude wild-type extracts are normally fairly low, thus accounting for the apparent stability of these extracts (2). During subsequent handling, the proteases in such extracts slowly become activated. This finding accounts for the difference in stability to heating of various aliquots of crude wild-type enzyme, depnding upon the length of exposure to higher temperatures. The greater the activation of the proteases, the more heat-sensitive will be the corresponding decarboxylase preparation ("old" enzyme, Fig. 3). The increased protection to heating conferred by increasing concentrations of phosphate buffers (Fig. 3) (2) must involve inhibition of protease A as well as protease B. Those preparations having higher levels of activated proteases ("old" enzyme, Fig. 3) would be expected to be denatured to a greater extent when heated in phosphate buffer of a given concentration than preparations in which the proteases were much less activated ("new" enzyme, Fig. 3).

Evidence has been presented supporting the concept that TPP complexes of higher aldehydes are more unstable than 2-hydroxyethyl TPP and tend to decompose spontaneously to free aldehydes (3). This finding can explain the results of Table V where it was shown that mutant decarboxylase, which had been degraded considerably by protease action, was, unlike wild-type decarboxylase, more active in decarboxylating higher α-keto acids than in decarboxylating pyruvate. These results provide strong confirmation for the thesis that the observed change in activity ratio during aging of mutant extracts represents a selective degradation of the second catalytic site of pyruvate decarboxylase. Since the first catalytic site is relatively unaffected by this proteolytic action (Fig. 6), it remains capable of forming aldehyde-TPP complexes from the various α-keto acids used (Table V). If the higher aldehyde-TPP complexes are inherently unstable, they would decompose spontaneously, and hence such an altered enzyme would be quite active in de-

carboxylating higher α-keto acids. The rate of decarboxylation of pyruvate by this altered enzyme would be expected to be relatively slower since 2-hydroxyethyl TPP is a stable compound (13), and would require further catalytic action (second site activity) to dissociate to free acetaldehyde.

Although not heretofore studied with pyruvate decarboxylase, the action of proteolytic enzymes on other enzyme proteins has been shown, in several cases, to result in partial degradation of these proteins without loss of their catalytic activity. A few examples of some recent studies of such phenomena include the demonstration by Motonage (18) that partial chymotryptic digestion of yeast cytochrome c results in an active protein fraction which has a different *N*-terminal amino acid (lysine instead of threonine), the modified cytochrome c having an altered chromatographic behavior. Givol *et al.* (19) have shown that β-galactosidase from *Escherichia coli* can be modified by treatment with several proteolytic enzymes and still retain enzymatic activity. Treatment of yeast hexokinase with trypsin converts the former enzyme to a more acidic form (20, 21) which is still catalytically active. The modified hexokinase is relatively unstable (20) and, unlike the native enzyme, which is a tetramer, appears to exist as a dimer (21). During tryptic digestion of hexokinase, there is a release of acid-soluble ninhydrin-positive material (20) indicating production of peptide fragments. The changes in hexokinase observed during tryptic digestion are similar to changes brought about by the action of proteolytic enzyme present in yeast (20), which has been found to contaminate recrystallized preparations of hexokinase (22). It has also been shown (23) that yeast contains a proteolytic enzyme (most probably protease B or protease C) which is capable of modifying crystalline yeast phosphoglyceric acid mutase so that four electrophoretically distinguishable and enzymatically active components are produced.

After completion of the experiments described in the present paper it was demonstrated that yeast extracts contain another protease (protease C) (6) which has several properties similar to those of protease B. Since protease C is not strongly inhibited by 1.0 M sodium phosphate or 50% glycerol (Table III) agents which exert good protective effects on pyruvate decarboxylase, it seems unlikely that this protease plays a role in the selective degradation of the decarboxylase. It is possible, however, that protease C may, like protease A, degrade pyruvate decarboxylase nonselectively. Further studies are now in progress to determine more precisely the action of the various yeast proteases on pyruvate decarboxylase.

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