*GENETIC ALTERATION OF PYRROLINE-5-CARBOXYLATE REDUCTASE IN NEUROSPORA CRASSA**

By Takashi Yura

DEPARTMENT OF MICROBIOLOGY, YALE UNIVERSITY SCHOOL OF MEDICINE, NEW HAVEN, CONNECTICUT

Communicated by E. L. Tatum, December 5, 1958

INTRODUCTION

There are several well-known cases in which genetic changes in microorganisms are associated with qualitative alteration of a specific enzyme.[1-3] In these instances, comparisons were made either between enzymes from a wild-type strain and those from a "revertant" strain secondarily obtained from one "lacking" a particular biosynthetic enzyme,[1,3] or between enzymes from different wild-type strains.[2] Recent studies of two cases of temperature-sensitive mutants of *Neurospora*, derived by one-step mutation from a wild-type strain, also revealed some evidence of enzyme alteration.[4, 5] Such studies with altered enzymes provide not only a basis for understanding the mechanism of gene mutation but also excellent tools for investigating the problems of gene action. This paper presents evidence that the enzyme pyrroline-5-carboxylate reductase in *Neurospora* undergoes an alteration, perhaps at the catalytic site, as a result of single-gene mutation at the *prol*-1 locus.

Pyrroline-5-carboxylate reductase has recently been obtained in cell-free extracts of *Neurospora* and has been characterized[6]. This enzyme catalyzes the terminal step of proline biosynthesis: a pyridine nucleotide-dependent reduction of Δ^1-pyrroline-5-carboxylate. A proline-requiring mutant strain, 21863,[7] is blocked at this step of the reaction and shows a greatly decreased reductase activity.[6] The low activity of the enzyme in mutant extracts does not seem to be due to the presence of substance(s) which might inhibit reductase activity in this strain. As will become apparent, this "residual" reductase of the mutant strain seems to have much higher activation energy and lower thermostability when compared to the enzyme of the wild-type strain.

MATERIALS AND METHODS

Enzyme Preparation.—Wild-type strain 74A[8] of *Neurospora crassa* and proline-requiring mutant strain 21863-6A[9] were used in most of the experiments. Mycelia were grown in 15-liter quantities of minimal medium (N),[10] either with or without 0.01 per cent L-proline, for 5 days at 30° C. with aeration. Extracts were prepared from the lyophilized mycelia and partially purified through the first ammonium sulfate fractionation in essentially the same way as described elsewhere.[6] The ammonium sulfate fractions of 25–45 per cent of saturation were collected in $M/10$ potassium phosphate buffer (pH 7) with 1 mM glutathione and dialyzed against the same buffer with glutathione. Enzyme preparations thus obtained could be stored at −15° C. for at least a week without appreciable loss of activity.

Enzyme Assay.—Enzymatic activity in cell-free extracts was assayed either by following the disappearance of reduced triphosphopyridine nucleotide (TPNH) in a Beckman spectrophotometer or by bioassay for L-proline, using a mutant

strain of *Escherichia coli*, according to the general procedures of Yura and Vogel.[6] Some modifications were made, however, in the bioassay procedures: the amount of TPNH used was increased to 2.5 μmoles per 1.2-ml. assay mixture, and the reaction period was shortened to 15–30 minutes, in order to minimize enzyme inactivation.

Heat Inactivation.—One milliliter amounts of enzyme solution containing 30 mg. protein were dispensed in 8-ml. test tubes. These tubes were placed in a water-bath at a constant temperature, shaken for 30 seconds, and then allowed to stand for the rest of the period of treatment. Inactivation was stopped by placing the tubes in ice, with shaking for the first 30 seconds. Heat-treated extracts as well as untreated control extracts were centrifuged at 10,000 $\times$ g for 20 minutes, and the resulting supernatants tested for enzymatic activity. Activity was usually measured by the optical method and confirmed by the bio-assay method. The values thus obtained were corrected for volume change due to the denatured protein in extracts.

RESULTS

No major differences were found between pyrroline-5-carboxylate reductase of the wild-type strain and that of the mutant strain with respect to stoichiometry, relative activity with the two pyridine nucleotides (TPNH and DPNH), effect of pH, affinity for the substrate, or fractionation behavior. However, the reductases of the two strains were strikingly different from each other with respect to the activation energy of the reaction and to the thermostability.

Activation Energy.—To study the effect of temperature on the rate of reduction of pyrroline-5-carboxylate, the reaction was run at several different temperatures (10°–35° C.) under otherwise standard assay conditions. The results of several such experiments are shown in Figure 1. Both the optical and the bio-assay methods

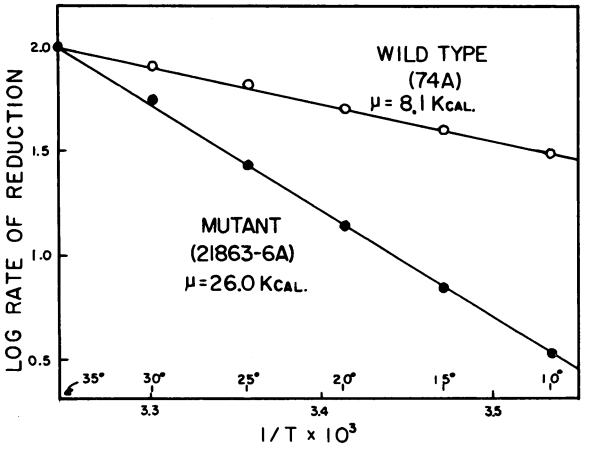

FIG. 1.—Arrhenius plot of the effect of temperature on the rate of reduction of pyrroline-5-carboxylate with extracts from wild-type and mutant strains of *N. crassa*.

were used for the rate determinations and showed quantitative agreement. Inactivation of enzyme during the incubation period was negligible. From the results presented, it may be calculated that the activation energies were about 8 and 26 kcal. per mole, respectively, for the reactions catalyzed by the enzymes in the wild-type and in the mutant extracts. This corresponds to a temperature coefficient Q_{10} of 1.6 for wild-type and 4.1 for mutant extracts.

In order to test for possible interaction between wild-type and mutant extracts, the effect of temperature on the reaction velocity was studied in the presence of a mixture of both extracts. Table 1 shows the results of such an experiment. The

TABLE 1*

EFFECT OF TEMPERATURE ON RATE OF REACTION CATALYZED BY WILD-TYPE AND/OR MUTANT PYRROLINE-5-CARBOXYLATE REDUCTASE OF *N. Crassa*

EXTRACT	ENZYME (UNIT)	PROTEIN (MG.)	L-PROLINE FORMED (μMOLE) AT 35° C.	25° C.	15° C.
Wild type.....	0.51	0.008	0.15	0.12	0.08
Mutant.....	0.25	2.700	0.18	0.07	0.02
Mixture.....	0.76	2.708	0.34	0.18	0.09

* The rate of reaction is expressed in terms of the amount of L-proline formed under standard assay conditions.

rate of reaction is expressed as amount of L-proline formed at varying temperatures under otherwise standard conditions. It can be seen that the reaction rate obtained with the mixed extracts represents the sum of the individual rates of each extract. Thus there is no evidence for interaction, and the observed difference in activation energy may be attributed to a structural difference between the enzymes, possibly at the catalytic site. It was further found that wild-type extracts, in which 50–90 per cent of enzymatic activity had been destroyed through heat treatment, exhibited activation energies similar to those of the fresh extracts. Hence no evidence has been obtained with the wild-type extracts employed to suggest the presence of more than one molecular species of reductase exhibiting different activation energies.

Thermostability.—Heat inactivation of the enzyme in either wild-type or mutant extracts follows the kinetics of a first-order reaction at several temperatures tested (40°–64° C.). Figure 2 shows the results of the experiments at 48° C. It was

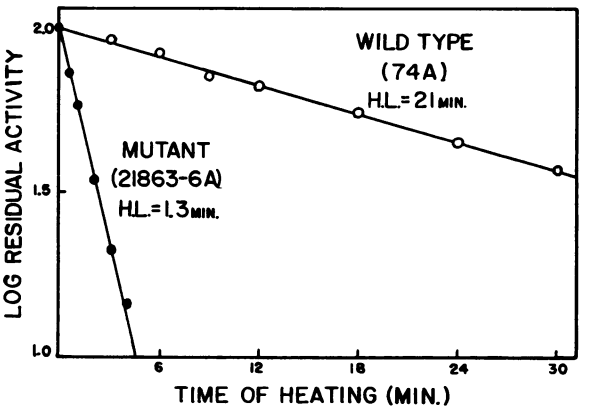

FIG. 2.—Inactivation of pyrroline-5-carboxylate reductase from wild-type and mutant strains of *N. crassa* by heat treatment at 48° C. *H.L.*: Half life.

found that the enzyme in the mutant extracts was much more labile to heat than that in the wild-type extracts, the half-life of the mutant enzyme at this temperature being 1.3 minutes, as compared to 21 minutes for the wild-type enzyme. Changes in concentration of the extract, with respect to either enzyme or protein, did not cause appreciable change in the thermostability of the enzyme: neither substitution of 99 per cent of *Neurospora* protein with bovine serum albumin nor tenfold dilution of extracts to a concentration of 3 mg. protein per ml. had any effect on the thermostability of the wild-type enzyme. A fivefold change in protein concentration

(10–50 mg. per ml.) made no difference in the thermostability of the mutant enzyme.

Enzyme inactivation by heat treatment at varying temperatures has been studied, and the results are shown in Figure 3. The time of heating was 3 minutes at each temperature. Here again, the enzyme in mutant extracts is seen to be much more labile than that in wild-type extracts in the temperature range tested. To examine the temperature dependence of heat inactivation quantitatively, the data in Figure 3 were used for an Arrhenius plot (Fig. 4) in which the logarithm of the

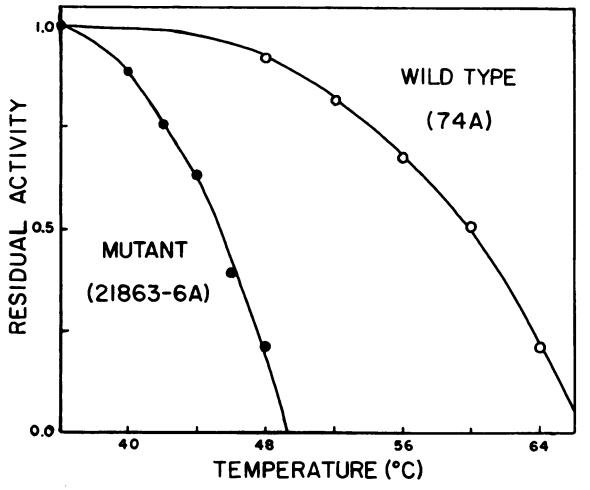

FIG. 3.—Inactivation of pyrroline-5-carboxylate reductase from wild-type and mutant strains of *N. crassa* by heat treatment at varying temperatures (40°–64° C.). Time of heating is 3 minutes at each temperature.

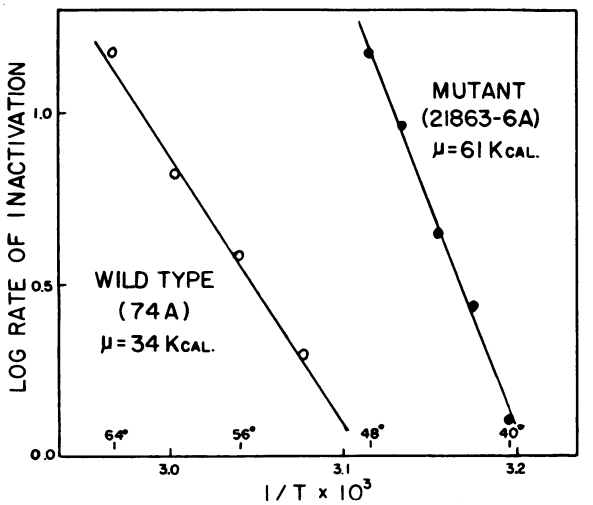

FIG. 4.—Arrhenius plot of the effect of temperature on the rate of heat inactivation of pyrroline-5-carboxylate reductase from wild-type and mutant strains of *N. crassa*.

rate of inactivation is plotted against $1/T$. It could be shown that inactivation of the mutant enzyme required about twice the energy as that of the wild-type enzyme, indicating the higher degree of temperature dependence of inactivation for the mutant enzyme.

Mixing experiments were then performed to test for possible interaction between wild-type and mutant extracts during heat inactivation. The results of a typical

TABLE 2

HEAT INACTIVATION OF PYRROLINE-5-CARBOXYLATE REDUCTASE FROM WILD-TYPE AND/OR MUTANT STRAINS OF *N. Crassa*

	Residual Enzymatic Activity after Exposure to 48° C. for			
Extract	0	3	6	24 (Min.)
Wild-type	25	24	22	16
Mutant	100	21	5	0
Mixture	128	43	30	15

experiment are shown in Table 2. It can be seen that the residual activity of the mixture after exposure to a temperature of 48° C. for varying periods is equal to the sum of the activities of the separate controls, suggesting that the rate of heat inactivation of the enzyme in either extract is not affected by the presence of the other. Hence the observed difference in thermostability also seems to reflect a structural difference between the enzymes rather than a difference in the amounts of destructive or protective agents in the extracts.

Growth Experiments.—In view of the unusually high activation energy obtained with the enzyme in the mutant extracts, it was thought that this might be reflected in the growth behavior of this mutant strain, if conditions were set so that the function of this enzyme would be the primary limiting factor of growth. Such an experiment was possible, since this mutant is capable of growing in minimal medium upon prolonged incubation, and this capability had been shown to depend on the residual enzymatic activity.[11] 125-ml. Erlenmeyer flasks, each containing 20 ml. of minimal medium inoculated with conidia of the mutant strain, were incubated at 30° C. When the cultures had reached early logarithmic phase (55–60 hours), half of the flasks were incubated further at 35° C. and the remainder at 25° C. Sample flasks were then taken out for dry-weight determination of mycelia at appropriate intervals, and growth rates were calculated by taking the reciprocal of the time in hours required for doubling the dry weight of the mycelium during the logarithmic phase. The results of such experiments are summarized in Table 3. The second column of the table represents the experiment described

TABLE 3*

GROWTH RATES OF WILD-TYPE AND PROLINE-REQUIRING MUTANT STRAINS OF *N. Crassa* AT 35° AND 25° C.

	Mutant		Wild-Type	
Temperature	No Proline	With Proline†	No Proline	With Proline†
35° C. (A)	0.125	0.170	0.195	0.200
25° C. (B)	0.062	0.128	0.155	0.165
A/B	2.02	1.33	1.26	1.21

* Procedures described in the text. Growth rates are calculated by taking the reciprocal of time (hour) required for doubling the dry weight of the mycelium during the logarithmic phase. The figures represent the average from five repeated experiments.

† One ml. of L-proline (2 mg/ml) was added to each flask at the time of the transfer. For the "no proline" controls, 1 ml. of water was added to each flask.

above, and the ratio of the growth rates at 35° and 25° C. under these conditions was about 2.0. This is significantly higher than the ratio obtained when the reductase is not the growth-limiting factor, as, for example, when proline is added to the minimal medium at the time of the transfer (third column, Table 3). The wild-type strain shows the ratios of 1.2–1.3 whether or not proline is present in the minimal medium.

The higher ratio of the growth rates of the mutant strain in minimal medium

can be explained in terms of the effect of temperature either on enzyme formation or on enzyme function. The former possibility is unlikely, though not entirely excluded, since the mycelia obtained after incubation at 35° or 25° C. showed the same specific activity of the reductase under the conditions employed. Hence these results may be taken as in vivo evidence for the higher activation energy of the mutant enzyme.

Genetic Analysis.—Since the wild-type strain 74A and the mutant strain 21863-6A so far used for comparisons are not isogenic, it was necessary to show that the observed differences in the enzyme are actually the reflection of the wild-type and the mutant allelic form of the *prol*-1 locus. Accordingly, the strain 21863-6A was crossed to a wild-type strain of the opposite mating type, and three complete asci were analyzed to follow the segregation patterns of the enzyme characteristics. Table 4 shows the results of this tetrad analysis. The strain 74-ORI-8a[12] used as a

TABLE 4

TETRAD ANALYSIS OF CHARACTERISTICS OF PYRROLINE-5-CARBOXYLATE REDUCTASE IN *N. crassa*: *74-ORI-8a* (*prol-1*$^+$) × *21863-6A* (*prol-1*$^-$)

Ascus No.	Spore Pair No.	*Prol*-1 Locus	Specific Activity (Unit/Mg Protein)	Activation Energy (Kcal/Mole)	Half-Life at 48°C. (Min.)
1	1	−	0.18	29.0	1.7
	2	+	93.0	8.0	22.0
	3	+	72.5	8.1	23.0
	4	−	0.20	27.0	1.5
2	1	−	0.28	24.0	1.5
	2	−	0.18	22.0	1.2
	3	+	88.5	8.2	22.0
	4	+	74.5	7.8	21.0
3	1	−	0.24	21.0	1.2
	2	−	0.27	26.0	1.3
	3	+	80.0	7.7	20.0
	4	+	82.0	7.3	22.0
Parents:					
74-ORI-8a		+	92.0	8.3	22.0
21863-6A		−	0.30	26.0	1.3

wild-type parent is essentially isogenic to the strain 74A. It is apparent from the table that both activation energy and thermostability of the enzyme segregate with the *prol*-1 locus. Furthermore, as shown in Table 5, several wild-type strains

TABLE 5

PYRROLINE-5-CARBOXYLATE REDUCTASE IN SEVERAL WILD-TYPE STRAINS OF *N. crassa*

Strain	Specific Activity (Unit/Mg Protein)	Activation Energy (Kcal/Mole)	Half-Life at 48° C. (Min.)
74A	84.5	8.1	21.0
74-ORI-8a	92.0	8.3	22.0
1A*	87.5	7.8	22.0
25a*	89.0	8.6	23.0
5256A*	82.5	8.3	19.0

* These strains were kindly supplied by Dr. Mary B. Mitchell.

which share the genetic background with the mutant strain used do not differ appreciably from one another with respect to the two characteristics of the enzyme. Thus there is no evidence for the participation of modifier genes at other loci which might affect the two enzyme characteristics to any great extent. It follows that the enzyme alteration appears to be a direct consequence of a mutation at the *prol*-1 locus.

DISCUSSION

Evidence that has so far been presented suggests that the pyrroline-5-carboxylate reductase of *Neurospora* undergoes a structural alteration, possibly at the catalytic site, as a result of a mutation at the *prol*-1 locus. Mixing experiments failed to give any evidence of interaction between wild-type and mutant extracts with respect to activation energy or to thermostability. The fact that enzyme inactivation by heat treatment follows the kinetics of a first-order reaction and that the activation energy of heat inactivation is in the range of typical values for protein denaturation also supports the possibility that the alteration involves the enzyme itself.

The unusually high activation energy obtained by in vitro experiments with the reductase of the mutant strain appears to be reflected in the high temperature coefficient of the growth of this strain when the growth depends primarily on the function of the reductase. The Q_{10} actually obtained under these conditions was about 2.0, which is still far below the value that might be expected from the in vitro experiments with extracted enzymes. This difference may be due at least partially to the thermolability of the enzyme in the mutant organism. It seems possible that the high activation energy associated with a specific enzyme in biochemical mutants, as found in the present study, may provide an explanation for some of the instances in which temperature-sensitive mutants exhibit specific growth requirements only when grown at lower temperatures.

Genetic analysis has shown that both activation energy and thermostability of the enzyme, presumably together with many other enzyme characteristics, are primarily determined by the allelic states of the *prol*-1 locus. The question of whether the changes in activation energy and thermostability are a result of one and the same change in the enzyme molecule cannot be answered at the present time. The relation between these changes in the enzyme characteristics and the low specific activity of the enzyme observed in the mutant strain also remains to be investigated. Although the nature of the enzyme alteration is not known, the alteration may be a relatively minor one, since the enzyme retains its catalytic function in the mutant organism. In any event, the final answer to the question of the mechanism of the alteration may be given by detailed chemical studies of the enzyme itself.

It has been known for some time that, at least in *Neurospora*, a gene change frequently leads to a quantitative alteration in the ability of the organism to perform a given biochemical reaction.[13] Recent work[11] with the same proline-requiring mutant strain used in the present study provided enzymatic and genetic evidence to support this notion and led us to believe that the majority of single-gene mutations do not result in a complete loss of a specific enzymatic activity. The present finding of an altered pyrroline-5-carboxylate reductase in the proline-requiring strain suggests that a qualitative enzyme alteration accompanied by a quantitative change in enzymatic activity may be a rather frequent consequence of single-gene mutations.

SUMMARY

Evidence is presented which suggests that a structural alteration of pyrroline-5-carboxylate reductase takes place, possibly at its catalytic site, following a single-

gene mutation at the *prol*-1 locus of *Neurospora crassa*. The alteration results in a three- to fourfold increase in activation energy of the reaction and a marked decrease in thermostability of the enzyme in the temperature range tested. In addition, heat inactivation of the mutant enzyme shows a higher degree of temperature dependence than does that of the wild-type enzyme. Several other characteristics of the enzyme examined remain essentially unaltered. The enzyme alteration is accompanied by a marked decrease in specific reductase activity, and it is suggested that a qualitative enzyme change may prove to be a rather frequent consequence of single-gene mutations in which activity of a specific enzyme is known to be very low or "lacking."

The author expresses his deepest appreciation to Dr. David M. Bonner for the helpful discussions and encouragement throughout the course of this study. Sincere thanks are also due to Dr. Henry J. Vogel for his invaluable advice in the initial stage of the investigation.

* An abstract of this paper has been published in *Proc. X International Congress of Genetics*, Vol. 2 (Montreal, 1958). This work has been supported by a grant from the Atomic Energy Commission, Contract No. AT-(30-1) 1017, to Dr. David M. Bonner.

[1] W. K. Maas and B. D. Davis, these PROCEEDINGS, **38**, 785, 1952.

[2] N. H. Horowitz and M. Fling, *Genetics*, **38**, 360, 1953.

[3] J. R. S. Fincham, *Biochem. J.*, **65**, 721, 1957.

[4] S. R. Suskind and L. I. Kurek, *Science*, **126**, 1068, 1957.

[5] N. H. Giles, C. W. H. Partridge, and N. J. Nelson, these PROCEEDINGS, **43**, 305, 1957.

[6] T. Yura and H. J. Vogel, *Biochim. et Biophys. Acta*, **17**, 582, 1955; T. Yura and H. J. Vogel, *J. Biol. Chem.* (in press).

[7] G. W. Beadle and E. L. Tatum, *Am. J. Bot.*, **32**, 678, 1945.

[8] This strain was kindly supplied by Dr. Patricia St. Lawrence.

[9] This strain is a leaky derivative of strain 21863, obtained by outcrossing the latter to the wild-type strain 74A. The strain was chosen because of its relatively high reductase activity (0.2–0.4 per cent of the wild-type level).

[10] H. J. Vogel, *Microbial Genet. Bull.*, No. 13, p. 42, 1956.

[11] T. Yura, thesis, Yale University, 1957; T. Yura, *Proc. Intern. Symposium on Enzyme Chemistry* (Tokyo and Kyoto, 1957) ("I.U.B. Symposium Series," Vol. 2 [Tokyo: Maruzen Co., 1958]), p. 377.

[12] This strain was kindly supplied by Dr. Frederick J. de Serres.

[13] D. M. Bonner, C. Yanofsky, and C. W. H. Partridge, these PROCEEDINGS, **38**, 25, 1952.

INDUCED ELECTROMAGNETIC FIELDS IN THE EARTH

BY JOHN CARSTOIU

TEXAS INSTRUMENTS INCORPORATED, DALLAS 9, TEXAS

Communicated by L. Brillouin, December 29, 1958

1. *Introduction.*—In addition to secular variation, the geomagnetic field is subject to daily variations in magnitude and character depending on geographic position, the seasons of the year, and disturbances of the sun. Notable changes in normal values also occasionally occur during strong storms, such as cyclones of electric charges. Generally speaking, there is a definite interaction between some