Effects of pH and Temperature on the Wild-Type and a Mutant Form of *Neurospora* Glutamate Dehydrogenase

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(Received 24 October 1966)

1. We confirm the observation of Bürk (1965) that *Neurospora crassa* NADP-linked glutamate dehydrogenase normally exists in an inactive form below pH 7.0 and in a fully active form above pH 8.0 in either tris or orthophosphate buffer. At pH 7.4 the enzyme is about half activated at 25°. 2. The variety of the enzyme produced by the mutant *am*²¹ shows a similar behaviour except that the transition is shifted about one pH unit in the alkaline direction. 3. The *am*²¹ enzyme has previously been reported to be activated by brief warming to 30° in phosphate buffer at pH 8.0. The wild-type enzyme shows a similar effect at pH 7.0. In tris buffer this effect is much less pronounced. 4. The *am*²¹ enzyme is extremely unstable at 47° at pH 7.0; its stability is somewhat greater at lower pH, and is markedly increased by increasing the pH in the range 7.0–8.7. The wild-type enzyme also shows an indication of a stability minimum at pH 7.0, but a temperature of 60° is needed for a measurable rate of inactivation. 5. The inactive form of the enzyme is much more subject to thermal irreversible denaturation than is the active form.

The *Neurospora crassa* mutant *am*²¹ has previously been shown (Fincham, 1957) to produce an abnormal variety of L-glutamate-NADP oxidoreductase (deaminating) (EC 1.4.1.4) (referred to below as glutamate dehydrogenase), which is largely in an inactive form at 20° in orthophosphate buffer, but which is very rapidly activated on warming to 30°. This activation is slowly reversed on cooling. Both the activation and the inactivation were shown to be first-order reactions, and it was concluded that a conformational change in the protein, without a change in its state of aggregation, was involved. The mutant enzyme could be activated even at 20° by incubation with a mixture of α -oxoglutarate and NADPH₂.

Until recently no comparable activation effects have been demonstrated with wild-type *Neurospora* glutamate dehydrogenase. It is now apparent that the reason for this is that enzyme assays have almost always been performed at pH 8.0 or 8.5, at which values the wild-type enzyme is strongly stabilized in its active form. However, Bürk (1965) has reported that the wild-type enzyme is in an inactive form in neutral and slightly acid solution. In this paper we confirm Bürk's (1965) observation and show that wild-type glutamate dehydrogenase resembles the

mutant enzyme in its behaviour, except that the pH at which the transition from active to inactive enzyme occurs is about 1 unit higher with the mutant. In a parallel study (West, Tuveson, Barratt & Fincham, 1967) it was shown that at pH 7.0 the wild-type enzyme shows striking activation by substrates, just as does the mutant enzyme at pH 8.0.

MATERIALS AND METHODS

Neurospora strains. Mutant *am*²¹ strains were the same as used previously (Fincham, 1957). The standard wild-type STA (also known as 74A) was used as the source of normal glutamate dehydrogenase.

Enzyme assays. Various assay systems were used and are specified in the legends of the relevant Figures. Rates of change of *E*₃₄₀ were measured on a Beckman DB spectrophotometer with a potentiometric recorder. Activities during enzyme purifications were measured in the assay system C of Fincham & Coddington (1963). Specific activities were expressed as 10³Δ*E*₃₄₀/min./protein unit. (One protein unit in 1 ml. at pH 7.4 gives *E*₂₈₀ 1.0, and is about 0.7 mg.).

Enzyme purification. Glutamate dehydrogenase was purified from both wild-type and mutant mycelia by the standard procedure used in this Laboratory (Fincham & Coddington, 1963). The *am*²¹ preparations were substantially homogeneous, as judged by polyacrylamide-gel electrophoresis, although some faint bands of contaminating proteins were visible. The wild-type enzyme used in the experiments reported in detail in this paper was obtained from the 'second-best' fractions from a final preparative

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column, and had about 60% of the specific activity (75000) of the best fractions (130000) that were judged to be almost pure. Less detailed experiments on other, purer wild-type preparations have given similar results.

RESULTS

Effect of pH of pretreatment on the activity of am^{21} enzyme. Fig. 1 shows the effect of holding the mutant enzyme at different pH values before assay. At pH 8.5 the enzyme is largely active, at pH 8.0 largely inactive, and at pH 8.2 about half activated. In the experiment illustrated the enzyme was pretreated at various pH values at 40° for 20 min., but varying temperature between 25° and 40° did not make any clear difference to the pH at which the transition between active and inactive enzyme occurred, at least when tris buffer was used. In phosphate buffer the effect of temperature at pH 8.0

Fig. 1. Effect of pH of pretreatment on activity of am^{21} enzyme. The purified enzyme (E_{280} 0.27) in 0.05M-phosphate buffer, pH 7.4, was diluted 12-fold into 0.1M-tris-chloride buffers to give the pH values indicated. After 30 min. at 40° 0.1 ml. samples were assayed in a system containing 2.5 ml. of 0.10M-tris-chloride buffer at either pH 7.5 (○) or pH 8.5 (●), 0.15 ml. of 0.2M- α -oxoglutarate (disodium), 0.15 ml. of ammonium chloride and 0.2 ml. of 0.4 mM-NADPH₂, at 30°.

is much more marked (Fincham, 1957). Although the pH of treatment before assay was critical, the pH of assay was not (Fig. 1). Apparently the enzyme, once activated, remained active in the presence of substrates during the few minutes of the assay even at pH 7.5. It was found in other experiments that, if the enzyme was fully active when added to the reaction mixture, the reaction was slightly faster at pH 7.5 than at pH 8.0, and 35% faster than at pH 8.7.

Effects of pH and temperature of pretreatment on the activity of the wild-type enzyme. Fig. 2 shows the effect on the activity on the wild-type enzyme of pretreatment for 11 min. at various pH values between 6.6 and 8.5. The shorter period of pretreatment, as compared with the 20 min. used in the experiment with am^{21} (Fig. 1) probably made no significant difference to the results since other experiments (e.g. that shown in Fig. 3) showed little further change in activity after the first 10 min. at 25° or 40°. As with the am^{21} enzyme, there is a transition from an active to an inactive state as the pH is lowered, but for the wild-type the point of 50%

Fig. 2. Effect of pH of pretreatment on activity of the wild-type enzyme. The enzyme fraction (E_{280} 0.47, estimated to be 50% pure) in 0.08 M-phosphate buffer, pH 7.4, was diluted tenfold into 0.1M-tris-chloride buffer (○) or 0.1M-sodium orthophosphate buffer (●, ●) to give the pH values indicated. After 11 min. at either 24.6° (○, ●) or 39.8° (●), 0.05 ml. samples were assayed in a system containing 2.6 ml. of 0.10M-tris-chloride buffer at pH 7.2, 0.15 ml. of 0.2M- α -oxoglutarate, 0.1 ml. of ammonium chloride and 0.1 ml. of 0.75 mM-NADPH₂, at 25°.

Fig. 3. Activation of wild-type enzyme at pH 7.0 with increase in temperature. Enzyme fraction (the same as under Fig. 2) from the refrigerator was diluted tenfold into 0.1M-sodium orthophosphate buffer and incubated at 21.5° (○), 24.6° (■), 30.6° (○) or 39° (●). At the times indicated 0.05 ml. samples were withdrawn and assayed as under Fig. 2.

inactivation is at pH 7.2–7.3 instead of at pH 8.1–8.2. The pH of transition was not strongly influenced by the temperature of pretreatment when tris buffer was used. In phosphate buffer, however, the midpoint of the transition was at about pH 7.3 at 25° and about pH 7.15 at 40°. In the range pH 7.0–7.4 merely warming the enzyme in phosphate buffer results in some degree of activation. Fig. 3 shows time-courses of the activation at various temperatures. These results closely parallel those obtained with *am*²¹ enzyme in phosphate buffer at pH 8.0 (Fincham, 1957).

Effects of pH on thermal irreversible denaturation. The glutamate dehydrogenase produced by *am*²¹ is extremely thermolabile (Fincham, 1957). To see whether the thermostabilities, as well as the activation properties, of the wild-type and *am*²¹ enzymes differed in their dependence on pH, the two enzymes were heated in 0.1M-phosphate buffer at various values between pH 5.5 and 9.0. To obtain easily measurable rates of denaturation a temperature of 47° was used for the *am*²¹ enzyme. With the wild-type enzyme a temperature of 59° was necessary for reasonably rapid denaturation. In no case did the denaturation process show more than small deviations from first-order kinetics, and the results are shown in Fig. 4 in terms of half-lives at the various pH values.

The *am*²¹ enzyme was clearly much less stable to heat at all pH values than was the wild-type enzyme. For the wild-type enzyme at 59° it was shown that, though stability was somewhat greater at pH 6 than at pH 7, it decreased sharply as the pH

Fig. 4. (a) Heat inactivation of *am*²¹ enzyme. Purified enzyme in 0.05M-phosphate buffer, pH 7.4 (*E*₂₈₀ 0.46), was diluted tenfold into 0.10M-sodium orthophosphate buffer to give the pH values indicated and heated at 47°. After 5, 10, 20, 30 and 40 min. of heating 0.1 ml. samples were withdrawn, mixed with 2.7 ml. of 0.1M-tris-chloride buffer, pH 9.0, and incubated at 37° for 20 min. to obtain complete activation of the surviving enzyme, before assay. The assay system contained 2.6 ml. of the enzyme-pH 9 buffer mixture, 0.2 ml. of 2M-monosodium L-glutamate and 0.2 ml. of 0.2% NADP, at 35°. The arrow indicates increasing stability with increasing pH; at pH 8.7, the half-life was several hours. (b) Heat inactivation of wild-type enzyme. The same enzyme fraction as for Fig. 2 was diluted tenfold into 0.1M-sodium orthophosphate buffer to give the pH values indicated and heated at 59°. After 10, 20, 40 and 60 min. of heating 0.05 ml. samples were withdrawn and assayed at 35° in the same assay system as under Fig. 2.

was further lowered. For both the wild-type and the *am*²¹ enzyme, increases in pH that result in conversion from the inactive into the active form

also cause a great increase in resistance to irreversible thermal denaturation. In another experiment predominantly inactive enzyme mixed with pH 7.9 buffer and immediately incubated at 47° lost one-third of its activity in less than 10 min.; the half-life of the remaining enzyme was about 3 hr. A similar experiment in which the enzyme was first activated at pH 8.9 showed no such initial phase of rapid loss of activity. All these observations are consistent with the view that the active form of the enzyme is more heat-resistant than the inactive form.

DISCUSSION

During recent years numerous reports have appeared of enzymes that undergo reversible transitions between active and inactive forms in response to shifts in pH [rabbit-muscle aldolase (Hass & Lewis, 1963)], or temperature [pyruvate carboxylase (Scrutton & Utter, 1964); nitrogen-fixing enzyme (Dua & Burris, 1963); glycogen phosphorylase (Graves, Sealock & Wang, 1965); muramidase (Hayashi, Hamaguchi & Funatsu, 1963)], or either pH or temperature [muramidase (Sophianopoulos & Weiss, 1964); yeast enolase (Rosenberg & Lumry, 1964)]. In several examples inactivation occurs in the cold and reactivation can be achieved on warming (Scrutton & Utter, 1964; Dua & Burris, 1963; Graves *et al.* 1965; Hayashi *et al.* 1963; Rosenberg & Lumry, 1964), and this kind of effect seems likely to be rather common. The study by Rosenberg & Lumry (1964) provides a particularly close parallel with the present case. At pH 5 and 25° yeast enolase is active; it can be reversibly inactivated either by lowering the pH to 4 or by refrigeration without changing the pH.

In several examples in the literature, reversible inactivation is accompanied by dissociation of an aggregate enzyme into sub-units (e.g. Scrutton & Utter, 1964). There seems to be good reason for discounting dissociation as an essential feature of

the present case. No effects of concentration on the rates of activation or inactivation have been observed (Fincham, 1957, and unpublished work). Further, A. Coddington & J. R. S. Fincham (unpublished work) have found that purified *am*²¹ glutamate dehydrogenase at a concentration of 2 mg./ml. in 0.05 M-phosphate buffer had almost the same *S* value at 14.5° (*S*_{20, w} 11.2 s) as at 32° (*S*_{20, w} 11.6 s), although the enzyme was largely inactive at the first temperature and largely active at the second.

It is clear that the properties of the *am*²¹ enzyme, which at first seemed to be unique, are due to a relatively slight distortion of a capacity for conformational transition that is characteristic of the wild-type glutamate dehydrogenase. It is likely that this property of the normal enzyme is of significance in relation to the regulation of enzyme activity (West *et al.* 1967).

During this investigation H.R.G. was the recipient of a U.S. Public Health Service Special Fellowship (1-F3-A1-23877-01) from the National Institute of Allergy and Infectious Diseases.

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