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EVIDENCE FOR CONFORMATIONAL CHANGES IN GRAPE CATECHOL OXIDASE

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Key Word Index—*Vitis vinifera*; Vitaceae; catechol oxidase; conformational changes; electrophoretic patterns.

Abstract—A rapid, 4–10-fold, activation of grape catechol oxidase by a short exposure to acid pH or urea is demonstrated. Activation was either reversible or irreversible, depending on length and type of treatment. The change in activity of the enzyme is due primarily to an increase in V_{max}, while the affinity for 4-methylcatechol decreases and that for O_2 increases. Activation occurs in intact chloroplasts as well as in a partially purified enzyme preparation. Activation was apparently due to conformational changes in the enzyme. O_2 concentration appeared to control enzyme activity, presumably by an O_2 induced conformational change. Irreversible activation was accompanied by changes in the electrophoretic mobility of the enzyme.

INTRODUCTION

IN A PREVIOUS paper we reported on some of the properties of a particulate catechol oxidase from grapes.[1] Among the features described for this enzyme was that it showed a multiplicity of bands on acrylamide gel electrophoresis. At least eight bands having catechol oxidase were observed. These bands fell into two groups—four bands which migrated far along the gel and four bands with a relatively slow rate of migration. Subsequent observations indicated that during prolonged storage or after treatment of the enzyme preparation with urea the electrophoretic pattern changed and only the slow running bands appeared.* We therefore decided to investigate this in greater detail, taking into account the possibility that changes in aggregation and/or conformation took place in the enzyme during storage and possibly during preparation.

RESULTS

The electrophoretic pattern of catechol oxidase, extracted from grape chloroplasts, and stored for 2 months at pH 5·0 in citrate buffer, 0·1 M is shown in Fig. 1a. Clearly, only the slow moving bands are present. In contrast, freshly prepared enzyme showed three of the fast migrating bands and two slow moving ones (Fig. 1b). If the same preparation was now kept for two days at pH 5·0 in citrate buffer, its electrophoretic pattern changed and the fast migrating bands weakened, while the slowest migrating band increased in activity and an additional slow band appeared (Fig. 1c). We therefore tried to prepare and extract enzyme from grape chloroplast, maintaining a slightly alkaline pH (7·2–7·5) at all stages of preparation. The electrophoretic pattern of this enzyme preparation is shown in Fig. 1d. It can be seen that only the fast and one of the slow migrating bands appear. Thus, clearly the kind of bands obtained is a function of the pH during preparation and storage of the enzyme.

* E. HAREL, unpublished.

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This led us to investigate the possible effect of pH on enzyme activity. Enzyme was prepared in the usual way, while maintaining the pH at all stages between 7·2 and 7·5. The preparation was now rapidly brought to pH 5·0 with 1·0 M citrate buffer and kept at this pH for various periods of time. It was then rapidly brought back to pH 7·0 and its activity measured. Enzyme kept at pH 7·0, at the same ionic strength as the treated one served as control. Typical results are shown in Fig. 2. It can be seen that the acid shock resulted in a rapid increase in activity at pH 7·0 with a half-time of about 0·5 min. Figure 2 also shows the subsequent decay of activity at the neutral pH. Activity decayed with a half-life of about 5 min. The activation of the enzyme and the subsequent decline of activity suggest that conformational changes take place in the enzyme upon exposure to a pH shock. This type of activation was quite reproducible. However, the degree of activation was somewhat variable from one enzyme preparation to another. In a few cases no reversion after pH shock could be obtained indicating that whatever changes had occurred in the enzyme were irreversible. Quite probably irreversible changes of this kind may cause the appearance of the slow moving bands after prolonged storage at acid pH.

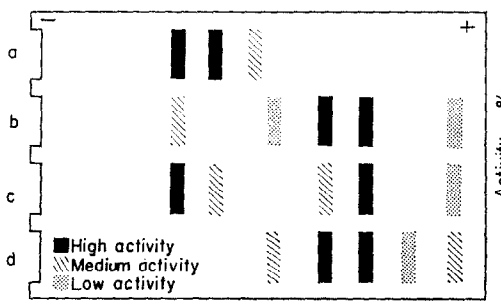

FIG. 1. ACRYLAMIDE GEL ELECTROPHORESIS OF GRAPE CATECHOL OXIDASE SHOWING DEPENDENCE OF BAND MULTIPLICITY ON pH DURING PREPARATION OR STORAGE OF THE ENZYME.

Enzyme extracted from lyophilized grape chloroplasts with Triton X100 and precipitated by $(NH_4)_2SO_4$ (40–95% saturation). Electrophoresis was performed for 3·5 hr at 350 V (20 V/cm) in 6% acrylamide in 0·02 M tricine buffer pH 7·9; (a) enzyme stored for 2 months in 0·1 M citrate buffer pH 5·0 at −20°; (b) freshly prepared enzyme; (c) enzyme stored for 2 days in 0·1 M citrate buffer pH 5·0 at −20°; (d) enzyme prepared as in (b) but the pH was maintained at 7·2–7·5 during precipitation with $(NH_4)_2SO_4$.

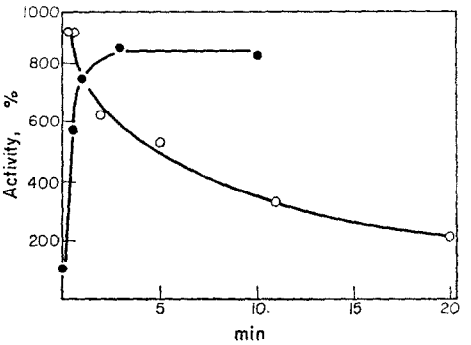

FIG. 2. ENHANCEMENT OF CATECHOL OXIDASE ACTIVITY AT pH 7·0 AFTER PREINCUBATION AT pH 5·0 AND SUBSEQUENT RELAXATION OF ACTIVITY.

Enzyme activity as % of activity before pH shock. ●—activity at pH 7·0 after preincubation for various periods of time at pH 5·0; ○—relaxation of activity during incubation at pH 7·0, following a 3 min shock at pH 5·0.

We also tested the stability of activity at acid pH with time. For periods of up to 3 hr at pH 3·4 in dil. phosphoric acid the enzyme preparation remained activated and showed typical reversion on being brought back to pH 7·0. Longer periods were not studied.

The pH shock involves a change in the ionic strength of the medium during treatment. For this reason the effect of ionic strength on enzyme activity was studied. Table 1 shows the effect of ionic strength on activity at pH 5·3 and 7·0, of catechol oxidase extracted from grape chloroplasts with Triton X100. It can be seen from Table 1 that at both pH values

enzyme activity increases with ionic strength of the buffer. This change in activity becomes very marked at high ionic strength. In subsequent experiments on the pH shock ionic strength was carefully maintained, so that during measurement of activity, final buffer concentration was 0·1 M. Under these conditions the pH shock and its reversibility were reproducible.

In order to follow the effect of ionic strength as a function of pH, enzyme was exposed to 90% saturation with $(NH_4)_2SO_4$ at pH 7·0 or 3·5 for 2·5 hr, then dialysed against 10^{-3} M phosphate buffer pH 7. Catechol oxidase activity at pH 5 and at 7 after pH shock remained more or less constant regardless of pretreatment, while activity at pH 7 before pH shock was increased by 800% by the concerted effect of high ionic strength and low pH. The electrophoretic pattern also changed; the fast moving bands disappeared and background staining became more pronounced (Fig. 3). A consequence of this irreversible activation was the loss of most of the pH shock effect.

TABLE 1. EFFECT OF IONIC STRENGTH ON ACTIVITY OF CATECHOL OXIDASE FROM GRAPES

Buffer concn (mM)	Activity at pH 5·3	Activity at pH 7·0
10	—	12
25	460	—
50	560	35
100	660	60
250	980	90
500	1260	120

Buffer: citrate-phosphate at equimolar concentrations. Substrate: 5 mM 4-methylcatechol. Activity in scale units per min.

TABLE 2. EFFECT OF PREINCUBATION WITH UREA ON GRAPE CATECHOL OXIDASE ACTIVITY

Length of preincubation (min)	Activity as % of control M urea 2	M urea 4
2	254	500
5	296	783
10	346	742
20	371	767

Enzyme was preincubated with 2 or 4 M urea in 0·1 M phosphate buffer pH 7·0. Activity was determined in 0·1 M phosphate buffer pH 7·0 with 5 mM 4-methylcatechol as substrate.

If the activation and subsequent decrease in activity are indeed due to conformational changes, it should be possible to induce similar changes in the catechol oxidase by means other than pH shock. For this purpose we selected treatment with urea (Table 2); it is clear that considerable activation of the enzyme results from incubation with urea. Constant activity was reached after about 5 min in 4 M urea. Activation with urea only occurred in the presence of phosphate buffer during treatment. Urea treated enzyme did not respond to a subsequent pH shock. The effect of urea was reversible. If the urea is diluted after 5 min of treatment, complete reversal of activation is obtained within 20 min. With longer periods of incubation in 4 M urea relaxation becomes more sluggish (Fig. 4).

Activation of an enzyme, due to conformational change, can occur either due to increase of the V_{max} or decrease of the K_m of the enzyme, or both. These parameters were studied in grape catechol oxidase before and after pH shock or urea treatment. Both pH shock and treatment with urea cause a marked increase in the K_m of the enzyme for its phenolic substrate, 4-methylcatechol, while increasing the V_{max} by at least 400%. It seemed possible that the pH and urea induced activation of the enzyme is due to a change in the affinity of the enzyme for oxygen. Since the pH shock is rapidly reversed, it was difficult to measure the change in K_m for oxygen following this treatment. We therefore selected the urea treatment

for studying the possible change in the K_m for oxygen. During this study we observed that the plot of V against oxygen concentration was distinctly sigmoid, with an inflection at about 1% oxygen (Fig. 5). Pretreatment of the enzyme with urea almost abolished the sigmoid part of the curve. At the same time urea treatment decreased the K_m of the enzyme for oxygen. It seems likely that the increase in activity of the enzyme after both urea and pH shock is due, at least in part, to the increased affinity for oxygen. The sigmoid nature of the curve is of the kind generally regarded as being characteristic of conformational changes in the enzyme. Such changes might be part of a mechanism controlling its activity.

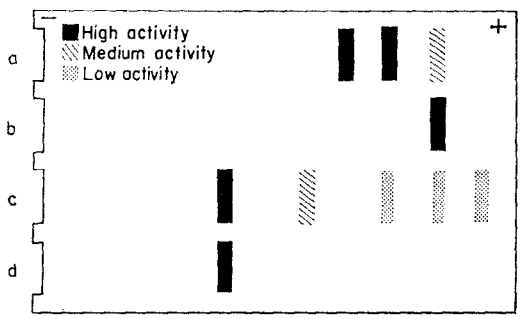

FIG. 3. ACRYLAMIDE GEL ELECTROPHORESIS OF GRAPE CATECHOL OXIDASE SHOWING THE EFFECT OF 90% SATURATION WITH $(NH_4)_2SO_4$ AT VARIOUS pH VALUES.
Enzyme extracted from lyophilized grape chloroplasts by 12 hr dialysis against 10^{-3} M phosphate buffer pH 7·0 after extraction of lipids by 90% acetone. The enzyme preparation was preincubated for 2·5 hr in 90% saturation with $(NH_4)_2SO_4$ and dialysed before electrophoresis was performed. (a) preincubation at pH 7·0, activity developed at pH 5·0; (b) preincubation at pH 7·0, activity developed at pH 7·0; (c) preincubation at pH 3·5, activity developed at pH 5·0; (d) preincubation at pH 3·5, activity developed at pH 7·0. Conditions of electrophoresis as in Fig. 1.

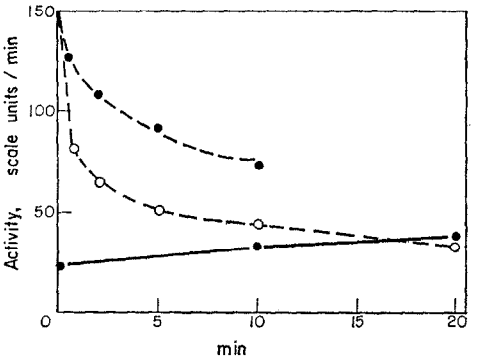

FIG. 4. RELAXATION OF CATECHOL OXIDASE ACTIVITY AFTER ACTIVATION BY UREA.
○——○ enzyme in 0·6 M urea after 5 min preincubation in 4 M urea; ●——● enzyme in 0·6 M urea after 2 hr preincubation in 4 M urea; ●—● enzyme in 0·6 M urea. All treatments were carried out in 0·1 M phosphate buffer pH 7·0.

DISCUSSION

Activation of catechol oxidases has been reported by a number of authors. Thus Kenten[2,3] reported on the latent broad bean tyrosinase and his studies have been extended by Robb *et al.*[4–6] and Swain *et al.*[7] Activation has also been reported for sugar beet catechol oxidase.[8,9] The phenomenon of latency has also been described for tyrosinase from animal

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tissues.[10–13] In some instances the activation seems to be due to conversion of the enzyme from an inactive to an active form.[11,13] However, the majority of the cases of activation were caused by denaturing agents of some kind such as acid, alkali, detergent or organic solvent treatment. In some cases activation was also induced by treatment with proteolytic enzymes.[9,12]

At the same time the multiplicity of form of catechol oxidase has been extensively documented.[14–18] However, in no case has activation been related to multiplicity. In addition most of the previous reports on activation have described irreversible processes resulting

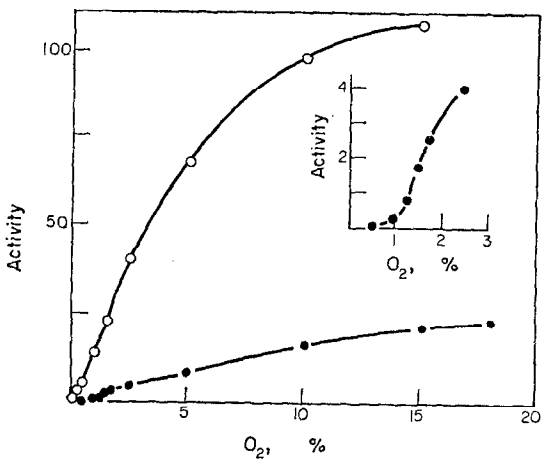

FIG. 5. THE EFFECT OF UREA ON THE DEPENDENCE OF CATECHOL OXIDASE ACTIVITY ON OXYGEN CONCENTRATION.

●—0·3 ml untreated enzyme; ○—0·05 ml enzyme preincubated for 2 min in 4 M urea. Activity determined in 0·1 M phosphate buffer pH 7·0 with 5 mM 4-methylcatechol as substrate. Activity is expressed in scale units per min. Inset shows activity of untreated enzyme at low oxygen concentrations on an expanded scale.

from short or long treatments. Swain *et al.*[7] suggest that activation is due to partial denaturation which in its early stages may be reversible. They also suggest that such activation may be due to limited conformational changes in the enzyme.

Our present results show that conformational changes occur in grape catechol oxidase upon either acid shock or urea treatment. These treatments apparently cause a conformational change near the active site of the enzyme resulting in a considerable acceleration of the rate of reaction. Although the rate of reaction is accelerated, the K_m for the phenolic substrate is increased while the K_m for oxygen is decreased at the same time. Rapid reversible

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conformational changes in proteins have been described for example for carbonylhemoglobin.[19] Acid catalysed structural changes have been correlated with changes in activity of lactic dehydrogenase[20] but in the latter case the acid induced transition was accompanied by a loss of enzyme activity. Similarly, the acid catalysed dissociation reaction which has been described for malate dehydrogenase led to reversible inactivation of the enzyme.[21]

That reversible acid catalysed conformational changes occur in proteins seems therefore well documented. The unusual feature of the grape catechol oxidase lies in the rapidity of the conformational change, its rapid reversibility and the fact that the conformational change leads to an activation of the enzyme. Reversibility of the acid activation of *Vicia faba* tyrosinase has been reported by Swain *et al.*[7] but in their case reversion was very slow. The majority of our experiments were made using partially purified enzyme prepared from freeze dried, isolated grape chloroplasts. However, the critical experiments were also made using intact freshly prepared chloroplasts. In this case too, the reversible pH shock was observed, as was the effect of oxygen on enzyme activity.

These results must be considered in relation to the effect of O_2 concentration on catechol oxidase activity. The effect of O_2 appears to be a typical conformational change,[22] where an enzyme becomes active only above a critical, albeit low oxygen concentration. The grape catechol oxidase, present in the chloroplast therefore shows two unusual features: control of activity by oxygen and a rapidly reversible activation due to changes in pH. Both the pH and the level of oxygen change in the chloroplast and the intact tissue, depending on the level of photosynthesis, activity of ion pumps and respiration. It thus seems not unlikely that catechol oxidase activity is in fact regulated *in vivo* by oxygen concentration and pH. This in turn may affect the level of free oxygen in the chloroplast. This at any rate appears to be a working hypothesis, which it should be possible to test experimentally.

The relation between conformational changes and multiplicity of enzyme forms must also be considered. The pH induced changes are much too rapid in both directions to assume that they simply involve monomer–polymer conversions in the enzyme. At the same time it appears that prolonged exposure to acid pH induces permanent irreversible changes in enzyme aggregation or conformation or both. These result in a considerable change in enzyme mobility during electrophoresis. It seems not unlikely that the grape enzyme also undergoes monomer–oligomer transitions and that the structure of the aggregate is dependent on pH and possibly also on ionic strength.

EXPERIMENTAL

White grapes were purchased at a local supermarket.

Preparation of lyophilized grape chloroplasts. Batches of 2 kg grapes in 200 ml of 1 M K_2HPO_4 containing 0·01 M Na ascorbate were homogenized for 15 sec at high speed in a Waring Commercial Blender. The suspension was filtered through 8 layers of gauze and centrifuged at 1000 *g* for 20 min. The precipitate was frozen at −20° and lyophilized and the dry powder stored at −20°.

Preparation of fresh chloroplasts. 200 g of grapes were crushed with 200 ml 0·5 M sucrose, 40 ml 1 M phosphate buffer pH 7 containing 0·01 M Na ascorbate. The suspension was filtered through gauze and centrifuged at 1000 *g* for 10 min. The precipitate was resuspended in 20 ml of 0·5 M sucrose 0·1 M phosphate buffer pH 7 and centrifuged at 1000 *g* for 10 min, again resuspended, centrifuged and resuspended in 0·5 M sucrose.

Triton X100 *solubilized enzyme.* 4 g of lyophilized grape chloroplasts, the equivalent of about 1 kg grapes, were suspended in 0·01 M phosphate buffer, pH 7 containing 1·5% Triton X100. After standing for 15 min

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at 2° the suspension was centrifuged for 1 hr at 15 000 *g*. The supernatant was subjected to $(NH_4)_2SO_4$ fractionation—the fraction precipitating between 45 and 95% saturation collected and redissolved. This solution after dialysis was used as enzyme source. Enzyme activity was determined by measuring oxygen consumption using a Clark polarographic electrode according to Mayer *et al.*[23]

pH shock treatment. Usually, to 0·1 ml of enzyme in 10^{-3} M phosphate buffer pH 7, distilled water was added to the required volume. Then, while mixing with a magnetic stirrer either 0·1 ml of 1 M citrate buffer at pH 5, for pH 5 shock, or 0·1 ml of 0·01 M phosphoric acid, for pH 3·5 shock, was added. After the appropriate time the pH was brought back to 7 with 0·2 ml of 1 M phosphate buffer pH 7. For determination of zero time activity after pH shock, the 0·2 ml of neutralizing buffer was injected together with the substrate into the reaction flask of the O_2 electrode.

Urea treatment. Freshly prepared 8 M urea was added to the enzyme preparation in the required amount as well as phosphate buffer to a final concentration of 0·1 M.

Acrylamide gel electrophoresis was performed as previously described.[1,24] The enzyme was located on the gels at the end of the run by dipping the gels for 5 min in appropriate buffer and then transferring them to 0·1 M citrate buffer pH 5 or 0·1 M phosphate buffer pH 7 containing 5 mM 4-methylcatechol and 1 mM *p*-phenylenediamine. Protein was determined according to Lowry *et al.*[25] 4-Methylcatechol was obtained commercially and recrystallized from *n*-hexane.

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