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Inactivation of Cytochrome-c with Glucose Oxidase

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A novel reaction of cytochrome-c from the horse heart with the enzyme glucose oxidase from *Aspergillus niger* (EC 1.1.3.4), in acidic media is described. Glucose oxidase is able to induce a rapid, profound and irreversible physico-chemical change in cytochrome-c, under anaerobic conditions and in the presence of glucose. The initial rate of reaction is almost independent of the concentration of enzyme and glucose. The striking feature of this reaction is the fact that the reaction proceeds efficiently even below a concentration of 10 nM enzyme.

Keywords: Cytochrome-c; Glucose oxidase; Inactivation

INTRODUCTION

Cytochrome-c is a soluble redox active hemoprotein localized in the inter-membrane space of mitochondria in eucaryotes. Release of cytochrome-c into the cytosol and its complexation with cellular proteins seems to be an essential step in initiating apoptosis or a programmed cell death.[1] Recently, apoptosis has received wide general attention, due to the work of Benner, Sulston, and Horvitz.[2] In addition, it has been found that the rabbit intestine alkaline phosphatase can induce a profound change in the spectral and physical properties of cytochrome-c from the horse heart, a phenomenon which was linked to apoptosis.[3,4] Also recently, it has been reported that polyanions Nafion and polyvinyl sulfate can induce spectral and physical changes in cytochrome-c, analogous to those induced by alkaline phosphatase.[5,6] A low molecular cyclic undecapeptide cyclosporine-A is also able to initiate cell death in mammalian cells, *in vivo* and *in vitro*.[7–9]

In this work, we report a novel reaction of cytochrome-c from the horse heart with glucose oxidase from *Aspergillus niger*. This bacterial enzyme induces a rapid, profound, and irreversible spectral change in the molecule of cytochrome-c, under anaerobic conditions and in the presence of glucose, at acidic pH. Many experiments are currently reported in the literature, in which cytochrome-c comes in contact with glucose oxidase in solution, for a short or a long period of time. This communication shows that, in such types of experiment, one must account for the slow destruction of cytochrome-c at neutral and a rapid destruction at acid pH values, and under anaerobic conditions as well. A possible connection of these phenomena with apoptosis is discussed below.

MATERIALS AND METHODS

Materials

Kinetic measurements were performed using glucose oxidase preparations from *Aspergillus niger* (EC 1.1.3.4), type VII-S, obtained from Sigma. The lyophilized powder contained approximately 80% protein the balance being phosphate buffer and sodium chloride. The catalase impurity in Sigma preparations was sufficient to rapidly remove the H_2O_2 product from solution. The specific activity of the enzyme was 100,000–200,000 units/gram solid (without added oxygen); one unit will oxidize 1.0 micromole of β-D-glucose to D-gluconic acid and H_2O_2 per minute, at pH 5.1 and 35°C. Glucose oxidase is a homodimer, composed of two identical subunits with the molecular weight of 150000 daltons. Each subunit carries one molecule of tightly bound coenzyme, FAD, which acts as a redox carrier in catalysis.[10] The concentration of

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enzyme active sites was determined from its molar extinction coefficient at 450 nm ($\varepsilon = 14100\,M^{-1}cm^{-1}$, in 0.1 M sodium acetate buffer pH 5.5) and expressed throughout this work as the concentration of its active sites.[10] Cytochrome-c from horse heart was obtained from Sigma and its concentration was determined from the molar extinction coefficient of ferricytochrome-c at 450 nm ($\varepsilon = 105000\,M^{-1}cm^{-1}$, at pH 7.0), or the difference between the spectrum of ferricytochrome and ferrocytochrome-c at 550 nm ($\varepsilon = 21000\,M^{-1}cm^{-1}$).[11]

Initial Velocity Studies

Initial velocity studies were carried out by measuring the absorption spectra using a double-beam spectrophotometer, in thermostated cuvette holders at 25°C. All reactions displayed in Figures 1–3 were performed in 3 ml-quartz cuvettes closed by a cap. The order of addition of reactants is indicated in the legends to the Figures. Usually, a buffer and a concentrated solution of glucose were mixed, followed by the addition of small aliquots of concentrated solutions of glucose oxidase and cytochrome-c. Throughout this work, 0.1 M McIlvaine citrate-phosphate buffers were used to maintain pH 2.9–6.5 in the media.

Anaerobic Conditions

All kinetic measurements presented in Figures 1–3, were performed anaerobically; the absence of oxygen was tested routinely with a Clark oxygen electrode (Hansatech Ltd., King's Lynn, England).

All experiments displayed in Figures 1–3 were performed in identical parallel runs, one in the spectrophotometer cuvette, and the other in the Clark oxygen electrode. The shape of the 3 ml-spectrophotometer cuvette is different from the geometry of the 3 ml-oxygen electrode. The cuvette is closed by a cap and the content is not stirred, except at the start of reaction; the reaction chamber of the oxygen electrode is closed, without much contact with oxygen in the air, and its contents are stirred continuously.[12] Nevertheless, the anaerobic conditions in the cuvette and in the oxygen electrode are very similar.

In each case shown in Figures 1–3, the comparison of reactions run in the cuvette and in the oxygen electrode, shows that oxygen disappears from the reaction mixture in cuvettes very rapidly, within seconds, which is in accordance with the data reported by Gibson *et al.*,[10] as well as with the well known catalytic properties of glucose oxidase.[13] The estimation of the concentration of dioxygen by the oxygen electrode is within 1% error. Thus, it is clear that, at relatively high concentrations of enzyme and glucose, anaerobic conditions are obtained in the spectrophotometer cuvette in all experiments displayed in Figures 1–3. However, when the concentrations of glucose or enzyme are very low, anaerobic conditions may not be met. In such cases, which are found in Figure 3A (the lowest concentration of enzyme) and in

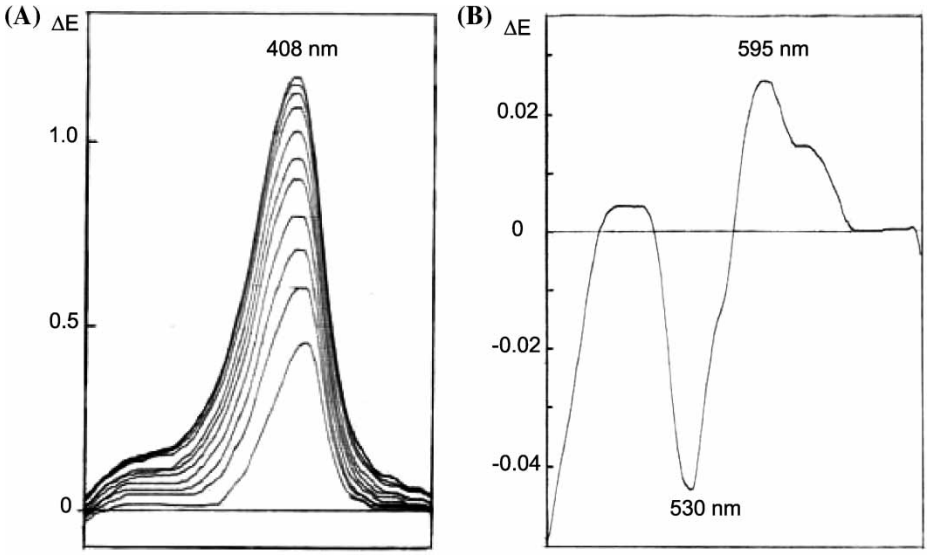

FIGURE 1 Spectral changes of cytochrome-c in the presence of glucose oxidase. (**A**) Spectral changes in the Soret region. Concentration and the order of additon of reactants was: buffer, glucose = 24.4 mM, cytochrome-c = 13.4 μM, glucose oxidase = 41.6 μM, at pH 2.9. The differential spectra were recorded at zero time (base line), 30 minutes (top spectrum), and times between. (**B**) Spectral changes in the 400–800 nm region. Differential spectrum of cytochrome-c in the presence and absence of glucose oxidase. Concentration and the order of additon of reactants was: buffer, glucose = 92.2 mM, cytochrome-c = 13.9 μM, glucose oxidase = 0.11 μM, at pH 2.9. The differential spectrum was recorded after 5 min. The spectral changes at 408 nm and 595 nm are in the opposite direction.

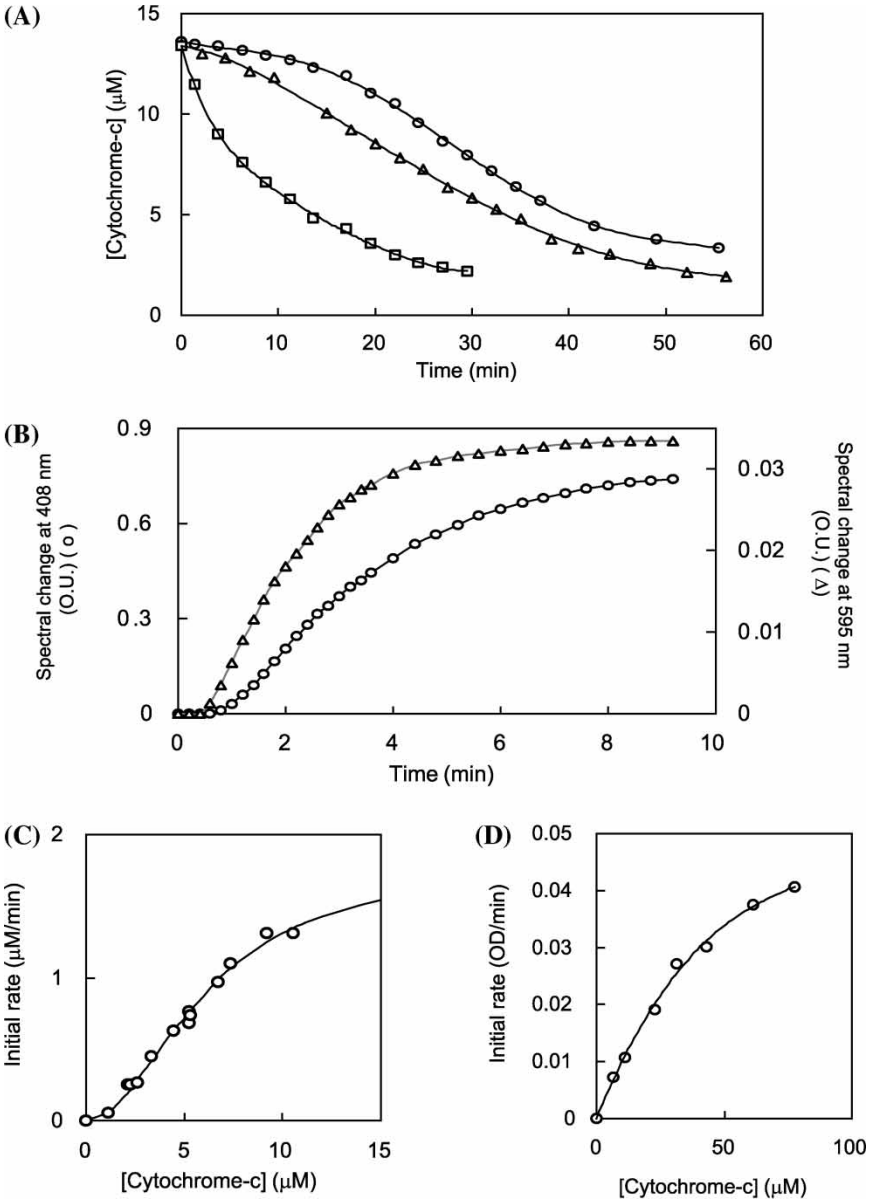

FIGURE 2 Influence of pH and substrate concentrations on spectral changes. (**A**) Influence of pH on the rate of spectral changes. Decrease in absorbancy of cytochrome-c (13.4–13.6 μM) at 408 nm in the presence of glucose (41.5 mM) and glucose oxidase (24.4 nM), at pH 6.50 (○), pH 3.86 (Δ) and pH 2.91 (□). (**B**) Reaction progress curves at 408 nm and 595 nm, at pH 2.91. The spectral change of cytochrome-c (10.4 μM) at 408 nm (○) and 595 nm (Δ) in the presence of buffer, glucose (86 mM), and glucose oxidase (86 nM). Note that the spectral changes at 408 nm and 595 nm are in the opposite direction. (**C**) Dependence of initial rates of reaction at 408 nm on cytochrome-c concentration. Cytochrome-c was mixed with buffer, glucose (24–97 mM) and the reaction was started with enzyme (1–10 μM) at pH 2.9. The initial rate of decrease in absorbancy at 408 nm was measured at different concentrations of cytochrome. Other experimental details are given in text. (**D**) Dependence of initial rates of reaction at 595 nm on cytochrome-c concentration. Cytochrome-c was mixed with buffer, glucose (103 mM) and glucose oxidase (105 nM) at pH 2.9. The initial rate of increase in absorbancy at 595 nm was measured at different concentrations of cytochrome. Other experimental details are given in text.

Figure 3C (the lowest concentration of glucose), the concentration of oxygen was not monitored by the oxygen electrode, because the difference in the shape of the spectrophotometer cuvette and the oxygen electrode must be taken into account under such limiting conditions.

Figure 2B shows that there is a short lag phase in the reaction, both at 408 nm and at 595 nm. For this reason, the initial rates of reaction in Figures 2B, 2C, and 3, were estimated at the steepest portions of the reaction progress curves and not at the beginning of reaction.

Data Processing

The saturation curve shown in Figure 2C, was fitted with a FORTRAN program of Cleland[14] (1979) to the Hill equation.[15]:

$$Initial\ rate = \frac{V_{max} \cdot [\text{ligand}]^n}{K_M^n + [\text{ligand}]^n} \quad (1)$$

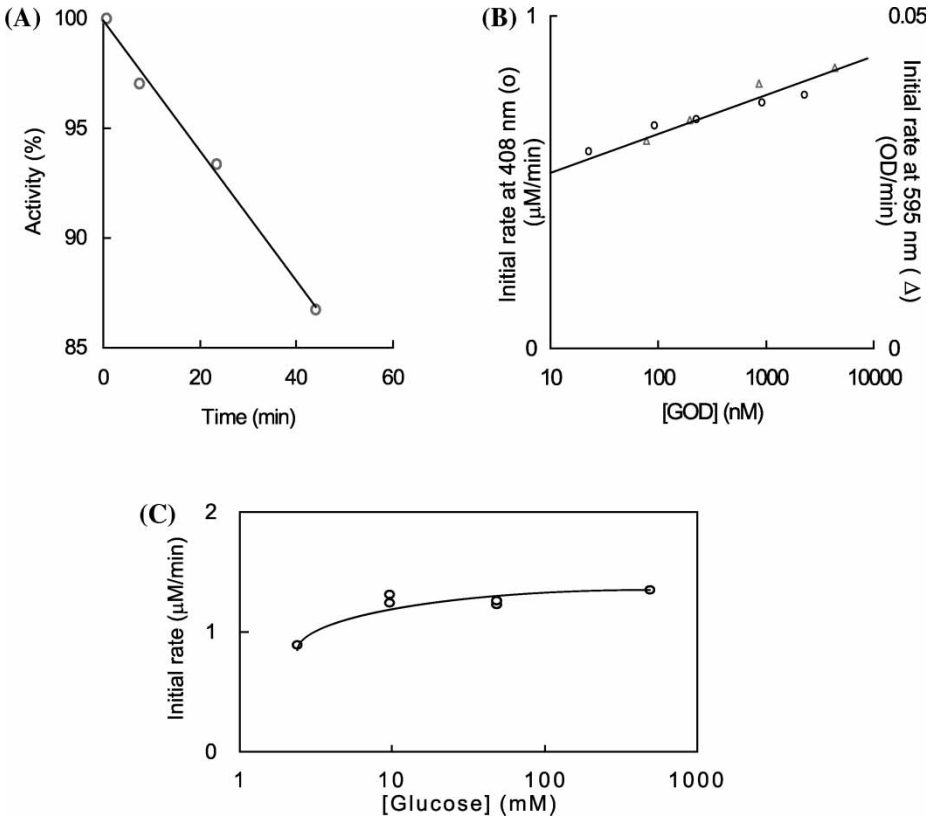

FIGURE 3 Dependence of initial rate of reaction on enzyme and glucose concentration. (**A**) Enzyme (42.2 μM) was incubated with, cytochrome-c (18.2 μM) and glucose (24.4 mM) at pH 3.9, and the activity of enzyme measured at times indicated. (**B**) Cytochrome-c (5.2 μM) was mixed with glucose (49 mM) and reactions were started with enzyme at pH 2.9; the initial rate of the decrease in absorbancy at 408 nm and the increase in absorbancy at 595 nm was measured at different concentrations of enzyme. (**C**) Influence of glucose on reaction rates at 408 nm. The concentration of cytochrome-c was 8.45 μM and glucose oxidase was 0.198 μM, at pH 3.0.

The saturation curve shown in Figure 2D, was fitted with a FORTRAN program of Cleland[14] to the following equation[15]:

$$Initial\ rate = \frac{V_{max} \cdot [\text{ligand}]}{K_M + [\text{ligand}]} \qquad (2)$$

The points in Figures 2C and 2D are experimentally determined values, while the curves are calculated from fits of the above equations to the experimental data.

RESULTS

In the presence of glucose oxidase and D-glucose, under anaerobic conditions, the spectrum of native cytochrome-c undergoes a rapid and profound change in acid media (Figure 1).

It appears that cytochrome-c undergoes a physical and physico-chemical changes under the influence of glucose oxidase. The spectral maximum of cytochrome-c at 408 nm decreases rapidly, reaching a very low value after a prolonged time (Figure 1A). A spectral change also takes place in the 400–800 nm region; a new split peak develops, with a maximum at 595 nm and 620 nm (Figure 1B). However, the spectral changes at 408 nm and 595 nm are in the opposite directions. At 550 nm and 695 nm, there are no spectral changes. The change at 550 nm will indicate a reduction of ferri to ferrocytochrome-c,[11] while the change at 695 nm is characteristic for an intact bond between heme and methionine-80;[11] the absence of spectral changes at both wavelengths indicates that both chemical processes do not occur.

The spectral changes of cytochrome-c in the presence of glucose oxidase are strongly dependent on pH. The rate of loss in absorbance at 408 nm increases rapidly with decreasing pH (Figure 2A). In Figure 1, the absorbance of cytochrome-c at 408 nm decreases with time, while the absorbance at 595 nm increases with time. The spectral changes at 408 nm and 595 nm are not simultaneous; the spectral change at 595 nm precedes the spectral change at 408 nm in time (Figure 2B). Since the spectral change at 408 nm is visibly lagging behind the spectral change at 595 nm, it appears that the physical change in the structure of cytochrome-c proceeds through at least two stages. In order to investigate the spectral changes at these two wavelengths more closely, the dependence of initial rates of spectral changes on the concentration of cytochrome-c was investigated at both wavelengths (Figures 2C & 2D).

Figures 2C & 2D show the influence of increasing concentrations of cytochrome-c on the initial rate of reaction. It again emphasizes the difference in response of reaction at 408 nm with respect to response at 595 nm. At 408 nm, the initial rate of reaction responds to increasing concentrations of cytochrome-c in a sigmoidal fashion (Figure 2C), while at 595 nm this response is hyperbolic (Figure 2D).

In the course of reaction, the enzyme remains active, with some loss of activity after a prolonged incubation time with cytochrome-c (Figure 3A). The initial rate of reaction, monitored from the decrease in absorbance at 408 nm, increases slightly with the concentration of enzyme (Figure 3B). The striking feature of this reaction is the fact that the initial rate of reaction is practically independent of the concentration of enzyme; even more striking is the fact that the reaction proceeds efficiently even at a very low concentration of enzyme, below 10 nM (Figure 3B). Reaction of cytochrome-c with glucose oxidase will not take place in the absence of glucose; however, the concentration of glucose in the reaction mixture has little influence on the initial rate of the reaction (Figure 3C).

The spectral changes similar to those shown in Figure 1, are also produced in the presence of polyethylene glycol in aqueous reaction mixtures in high concentrations (70–80%), under aerobic conditions and at low pH (data not shown); these observations will be elaborated in a forthcoming publication.

DISCUSSION

Mechanism of Reaction

Cytochrome-c is degraded in the presence of glucose oxidase under anaerobic conditions (Figure 1). The degradation of cytochrome-c by glucose oxidase is strongly accelerated by lowering the pH (Figure 2A).

Ferricytochrome-c has several conformational states in aqueous solutions; one of the conformational transitions takes place in acidic media with an apparent pK_a of 2.5.[11] The NMR spectrum of this state is that which would be expected from a high-spin iron interacting with weak field ligands such as solvent water.[16] A reversible conformational transition between these two states (one stabilized above pH 2.5 and the other below pH 2.5), apparently results from the ionization of Histidine-18, with an abnormally low pK_a of 2.5 induced by its hydrophobic environment. Thus, it appears that the spectral changes induced by glucose oxidase are coupled to the conformational change in protein structure, exposure of Histidine-18 to the solvent and a shift of its pK_a to alkaline, which may be the main cause for the subsequent gradual disruption of chemical bonds between the metal ion and the protoporphyrin ring.

The pH-induced conformational change is followed by the chemical change in the structure of cytochrome-c; it appears that the chemical change is initiated by the conformational change. Kinetic investigations, reported in this work, indicate that the chemical change, the degradation of cytochrome-c, proceeds by at least two steps. In the first stage, the spectral changes are taking place in the 570–750 nm region. In the second stage, an absorption band of cytochrome-c at 408 nm is lost (Figure 1). This Soret band is associated with the integrity of the heme structure, and the loss of this band indicates that the chemical bonds between the metal ion and the protoporphyrin ring of cytochrome-c are completely disrupted.[11] All attempts to locate the reduced form of cytochrome-c during the course of the reaction have failed; even under a wide variety of experimental conditions, we were unable to observe the typical absorption band of ferrocytochrome-c at 550 nm. Further, at 695 nm, there are no spectral changes. The change at 695 nm is characteristic for the integrity of the bond between heme and methionine-80,[11] and the absence of a spectral change at this wavelength indicates that the disruption of this chemical bond does not occur.

Thus, it appears that the pH-induced conformational change triggers a two-step chemical change. In the first step, the spectral changes are taking place in the 570–750 nm region, and in the second stage the chemical bonds between the metal ion and the protoporphyrin ring of cytochrome-c are disrupted. The reduction of cytochrome-c during the course of reaction does not occur.

The main question remaining is: what is the primary cause of reactions measured in this work, in the first place? Although the concentration of dioxygen in reaction mixtures is always low, some residual concentration may be present, which cannot be detected by the oxygen electrode. Also, low concentrations of hydrogen peroxide and superoxide anion radicals may be present, due to the catalytic activity of glucose oxidase.[13] Cytochrome-c is a well known radical trap for superoxide anion radicals.[17] Thus, the primary cause for the destruction of cytochrome-c may be the presence of minute amounts of oxygen, and consequently the presence of minute amounts of hydrogen peroxide or superoxide anion radicals in reaction mixtures.

Significance for Biochemistry and Medicine

The observations reported in this work are purely phenomenological; none the less, they are of

substantial practical importance for biochemistry and medicine.

First, reactions described above are taking place only in the presence of glucose oxidase and glucose, which implies anaerobic conditions. Glucose oxidase is extremely effective in an acidic milieu; already a few nanomoles of enzyme per liter are sufficient to produce a rapid degradation of cytochrome-c at pH 2.9 (Figure 3B) and it should not be forgotten that the enzyme is active even below pH 3.[13] This observation is of considerable interest to biochemistry and medicine. Many experiments are reported in the literature, in which cytochrome-c comes in contact with glucose oxidase in solution, for a short or a long period of time. This communication shows that, in such types of experiment, the slow destruction of cytochrome-c at neutral and its rapid destruction at acid pH values must always be accounted for.

Second, the medicinal significance of reactions described in this work are important, especially with regard to therapeutic properties of glucose oxidase and with respect to preclinical trials with this enzyme, reported some time ago.[18] Although information from the preclinical trials with this enzyme are not available, the experimental data reported in this work indicate that glucose oxidase may have some potentially toxic effects.

Third, an extremely efficient destruction of cytochrome-c by glucose oxidase, described in this communication, may also indicate one of the possible mechanisms that regulate apoptosis. In eucaryotes, the regulation of tissue cell numbers is a critical homeostatic objective that is achieved through tight control of apoptosis, mitosis and differentiation. While much is known about the genetic regulation of cell growth and differentiation, the molecular basis of apoptosis is less well understood.[19] Genes involved in both cell proliferation and apoptosis reflect the role of some stimuli in both of these processes, the cell response depending on the overall cellular milieu. Under pathological conditions, apoptosis accounts for regression of tissue hyperplasia once the stimulus to hyperplasia is removed. Spontaneous apoptosis in tumors may account for substantial cell loss and *retardation of tumor growth* while cells damaged by radiation, cancer therapeutic agents, and certain types of toxins often undergo apoptosis.[20–22] Such cell damage may be caused by glucose oxidase due to the reactions described above; thus, glucose oxidase may have potentially therapeutic effects in tumor cells. However, the involvement of glucose oxidase in apoptosis is speculative and the proposed involvement of the reactions described above requires much more theoretical and experimental support to be warranted.

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