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# Conformational Studies of Soluble and Immobilized Frog Epidermis Tyrosinase by Fluorescence

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## ABSTRACT

Fluorescence spectra and soluble quenching of intrinsic protein fluorescence were used as indexes of conformational changes suffered by frog epidermis tyrosinase. The activation process and the immobilization of the enzyme involving either free amino groups or its carbohydrate moiety were studied. The conformational changes resulting from denaturation of each one of the protein derivatives, as well as the effect of active center copper extraction, were followed by fluorescence studies.

The results showed that: (a) both activation and immobilization were accompanied by conformational changes of the protein leading to more unfolded states; (b) neither enzyme nor immobilized enzyme were fully unfolded upon denaturation although enzymic activity was lost; (c) the enzyme immobilized through its carbohydrate moiety was more unfolded upon denaturation than the enzyme immobilized through amino groups, thus pointing to a higher conformational stabilization in the last situation; and (d), that tryptophyl residues moved to a localization near the active site upon activation.

**Index Entries:** Conformational studies, of tyrosinase; tyrosinase, conformational studies by fluorescence; immobilized tyrosinase, fluorescence studies of; frog epidermis tyrosinase, fluorescence studies of; epidermal tyrosinase, fluorescence studies of frog; fluorescence studies, of frog epidermis tyrosinase; fluorescence studies, of frog epidermis tyrosinase.

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## INTRODUCTION

Frog epidermis tyrosinase (monophenol mono-oxygenase, EC 1.14.18.1) is a copper-containing enzyme that is extracted as a zymogen. The activation process has been studied by several authors, and it seems to involve a small peptide release and an unfolding of the protein chain (*1–4*). Tyrosinase both in proenzyme and enzyme forms has been immobilized in several solid supports in order to obtain further data about the difference between these forms, and about the activation process itself (*5–7*). Although the resulting immobilized proenzyme (IMP) and enzyme (IME) derivatives showed similar fluorescence spectra and kinetic parameters, there was a remarkable difference in the operational stability of the former derivatives; this result has been assigned to a distribution and number of linkages between the protein and the support that was different for the proenzyme and the enzyme, respectively, reflecting the fact that the two forms of tyrosinase must have unequal conformations. Tyrosinase is a tetramer, and when its subunits were immobilized, the spectral characteristics of the derivatives for proenzyme and enzyme were so different that it was necessary to conclude that the main difference between them was a conformational distinction in the respective subunits (*8*).

In order to obtain information about the conformational differences between proenzyme and enzyme forms, and to study the effect of immobilization on the protein conformation, soluble quenching of intrinsic protein fluorescence was used. It is a dynamic method that can yield information regarding the exposure of protein-bound intrinsic or extrinsic fluorophors (*9*). The technique has been widely used to assess the degree of exposure of tryptophyl residues in soluble proteins (*9–11*), but there is no known references about its utilization on immobilized enzymes.

## EXPERIMENTAL

### Materials

Frogs (*Rana esculenta ridibunda*) were obtained from local suppliers. Trypsin (EC 3.4.21.4) and $NaBH_4$ were purchased from Sigma Chem. Co. (St. Louis, MO, USA). Sepharose 4B, CM-Sephadex C-50, and Phenyl-Sepharose were obtained from Pharmacia Fine Chem. (Uppsala, Sweden). All the remaining chemicals were Merck analytical grade, and they were used without further purification.

### Methods

#### Tyrosinase Extraction and Purification

Frog epidermis tyrosinase was obtained as the proenzyme and purified by methods published elsewhere (*5,8*). Activation of the purified

purified proenzyme and enzymic activity measurements were carried out as in Iborra et al. (*12*).

### *Enzyme Immobilization*

Sepharose 4B was activated with cyanogen bromide (CNBr), as described by Axen and Ernback (*13*). The procedure for direct immobilization of tyrosinase on CNBr-activated Sepharose, the determination of coupled protein, and the measurement of the activity of the resulting derivatives, has been published elsewhere (*5,6*).

The enzyme was also immobilized in CNBr-activated Sepharose 4B via its carbohydrate moiety by the method of Hsiao and Royer (*14*) with minor modifications: to 40 mL of enzyme solution (80 μg/mL), sodium metaperiodate was added to achieve a final concentration in iodate of 0.02*M*, and the solution was kept 1 h in the dark. Iodate was removed by dialysis against water overnight. The enzyme was further modified by mixing it with ethylenediamine (0.05*M* final concentration) followed by the addition of 10 μL of 1*M* $NaBH_4$ at 30 and 60 min intervals. After 90 min the derivative was dialyzed against 5 L of 0.1*M* phosphate buffer, pH 7.0. The modified enzyme was coupled to CNBr-activated Sepharose 4B as the unmodified enzyme was. All the operations for the modification were carried out at 3–5°C and using 0.1*M* phosphate buffer, pH 7.0, as a medium.

### *Copper Extraction and Holo-Enzyme Reconstitution*

Soluble apo-tyrosinase was obtained by treating it with 10 m*M* KCN for 30 min. Then, the apoenzyme was washed in an Aminco CF-25 ultrafilter with 0.1*M* phosphate buffer, pH 7.0, until no $CN^-$ was detected in the ultrafiltrate. The apo-form of immobilized enzyme was obtained by pumping (20 mL/h), through a column with the immobilized derivative, a 10 m*M* KCN in 0.1*M* phosphate buffer, pH 7.0, solution for half-an-hour. The apoenzyme was thoroughly washed with 0.1*M* phosphate buffer, pH 7.0. None of the apo-derivatives showed dopa-oxidase activity.

Soluble and immobilized apo-tyrosinase recovered all their initial activity when treated with 0.1 m*M* $CuSO_4$ for 1 h.

### *Tyrosinase Denaturation*

Soluble tyrosinase was denatured when incubated with 3*M* guanidinium chloride, while both forms of immobilized tyrosinase needed a 6*M* concentration of the denaturing agent (*8*) in the same conditions (10 min of treatment at room temperature). Soluble and immobilized tyrosinase were also denatured by 8*M* urea if 30 m*M* mercaptoethanol and boiling were included in the treatment. Urea alone (8*M*, overnight at room temperature) failed to denature tyrosinase.

*Fluorescence Measurements*

Fluorescence measurements were made on a Perkin-Elmer MFP- 44B spectrofluorimeter, equipped with a differential corrected spectra unit Hitachi DSCU-2. All the spectra were corrected and performed at 25 ± 0.1°C, with 6 nm excitation and emission slits. In quenching experiments, an excitation wavelength of 300 nm was routinely used to ensure that the light was absorbed entirely by tryptophyl groups. Protein concentration of samples ranged from 13 to 38 μg/mL. The amount of immobilized derivative was chosen as low as possible in order to minimize the light scattering caused by Agarose beads. All solutions were filtered prior to use through a Millipore GSWPO 1300 filter, pore size 0.22 μm, in order to remove any other scattering particles.

*Quenching Experiments*

The fluorescence of the protein was quenched by the progressive addition of small aliquots of an 8*M* acrylamide solution. Iodide quenching measurements were made on solutions of a given protein concentration containing increasing amounts of KI (0–0.27*M*). Sodium chloride was used to keep the ionic strength constant. A small amount of $S_2O_3^{2-}$ ($10^{-4}$ *M*) was added to the iodide solution to prevent $I_3^-$ formation (*15*). No corrections for iodide nor acrylamide were necessary when a 300 nm excitation wavelength was used (*9*). Quenching results were plotted according with the classical Stern-Volmer equation (*11, 16*). When direct Stern-Volmer plots resulted downward curved as a consequence of high tryptophan heterogeneity, the modified Stern-Volmer relationship, valid for low quencher concentrations, was used (*11, 16*). Full emission spectra were recorded in order to detect any change in emission $\lambda_{max}$ that could be produced upon quenching.

## RESULTS AND DISCUSSION

### *Emission Spectra of Soluble Tyrosinase*

Figure 1 shows the corrected spectra of soluble proenzyme and enzyme forms. There were differences in maximum wavelength and intensity of the peaks providing the same protein concentration was used, either with excitation at 275 or 300 nm as shown by Fig. 1 and Table 1. The results can be explained in accordance with previous data (*3*), which showed that the activation process of tyrosinase changes the protein conformation. The change of maximum emission wavelength upon activation to a lower value accounts for a displacement of the tryptophyl residues to a less hydrophylic microenvironment (*17*). The increase of emission intensity upon activation can be ascribed to the displacement of tyrosyl emission maximum to a longer wavelength, and to its increased contribution to the overall fluorescence of the protein. Both effects can take place if the tyrosyl residues move from the inner protein core to a

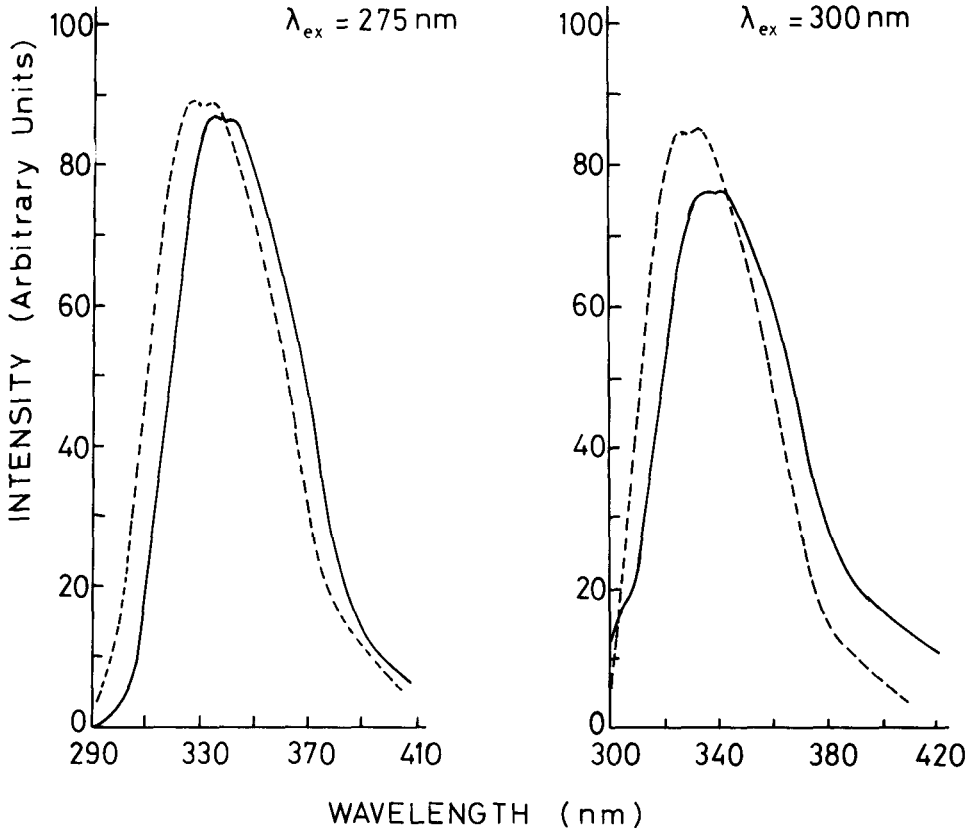


Fig. 1. Corrected fluorescence spectra of soluble proenzyme (solid line) and enzyme (dotted line). Protein concentration: 35 μg/mL in 0.1*M* phosphate buffer, pH 7.0, and 20°C. Enzyme activity: 16 U/mg. (A) Excitation wavelength: 275 nm; (B) excitation wavelength: 300 nm.

more exposed situation. Bearing in mind that the amount of tyrosyl and tryptophyl residues is lower for enzyme than for proenzyme as a result of proteolytic activation (*18*), the lack of shielding of tyrosyl residues caused by this activation unfolding ought to be considerable.

TABLE 1
Maximum Emission Wavelength and Half-Band Width of the Fluorescence Spectra Obtained for Tyrosinase in Different Forms[a]

| Tyrosinase form | $\lambda_{ex}$, 275 nm $\lambda_{em}$ | Width, nm | $\lambda_{ex}$, 300 nm $\lambda_{em}$ | Width, nm |
|---|---|---|---|---|
| SP | 332–337 | 56 | 333–340 | 55 |
| SE | 326–332 | 56 | 327–340 | 56 |
| $SP_D$ | 336 360 | 64.5 | 340 364 | 62 |
| $SE_D$ | 340 370 | 66.5 | 340 364 | 65.5 |
| IME | 309 335 | — | 335 | 78 |
| $IME_c$ | 309 336 | — | 337 356 | 79 |

[a]Excitation was carried out both at 275 and 300 nm (slit 6 nm).

Since the fluorescence spectrum of soluble proenzyme remained unchanged independently of the excitation wavelength used (275 or 300 nm), tryptophyl residues must be solely responsible for that emission. The lack of tyrosyl signals with excitation wavelength of 275 nm showed that energy transfer must have taken place (*19*), resulting in an increased emission intensity.

The appearance of a small splitting in emission spectra both for SE* and SP must be caused by tryptophyl heterogeneity in the protein.

### *Emission Spectra of Immobilized Tyrosinase*

Independently of the immobilization method used, the emission spectra were similar for the immobilized derivatives, and different from those of the soluble forms. First of all, as shown in Fig. 2a, two clear peaks were registered whose maxima appeared at 305–310 and 339 nm ($\lambda_{ext}$ = 275 nm), suggesting a separate contribution of tyrosyl and tryptophyl residues, and the decrease of energy transfer by an increase in the distance between tyrosyl and tryptophyl residues. Second, just one peak could be assigned to tryptophyl fluorescence with excitation at 275 nm. Since the $\lambda_{max}$ shifted towards longer wavelengths, two conclusions could be drawn: (1) there was a higher homogeneity, and the new microenvironment of tryptophyl residues had to be more hydrophylic than for the soluble enzyme. When excitation was carried out at 300 nm (Fig. 2b), a similar shift was observed, as well as a notable increase in half-band width closely similar for IME and IMEc (Table 1).

All these results claimed for an important conformational change of tyrosinase upon immobilization, to a more unfolded state, just as activation of soluble tyrosinase was accomplished by an unfolding of the protein chain (*3*).

### *Denatured Tyrosinase Fluorescence*

Under denaturing conditions, the fluorescence spectra of most proteins shifts to lower energy (approximately 350 nm) because of the exposure of buried tyrosyl and tryptophyl residues to the aqueous solvent, and to lower intensity because of the resulting increase in collisional quenching (*20*). As shown in Fig. 3, a distinctive tyrosyl emission was observed when the protein was denatured, thereby resulting in reduced energy transfer to tryptophyl residues because of the increase in the distance between these residues. When both SP and SE were incubated with 3*M* guanidinium chloride, the enzymatic activity was lost, but the appearance of the tyrosyl emission just as a shoulder, and two peaks for tryptophyl fluorescence, suggested an incompletely unfolded state.

*Abbreviations: SP, soluble proenzyme; SE, soluble enzyme; $SP_D$, denatured soluble proenzyme; $SE_D$, denatured soluble enzyme; IME, enzyme immobilized in CNBr-activated Sepharose 4B through primary amino groups; $IME_c$ enzyme immobilized in CNBr-activated Sepharose 4B through its carbohydrate moiety; SDS, sodium dodecyl sulfate.

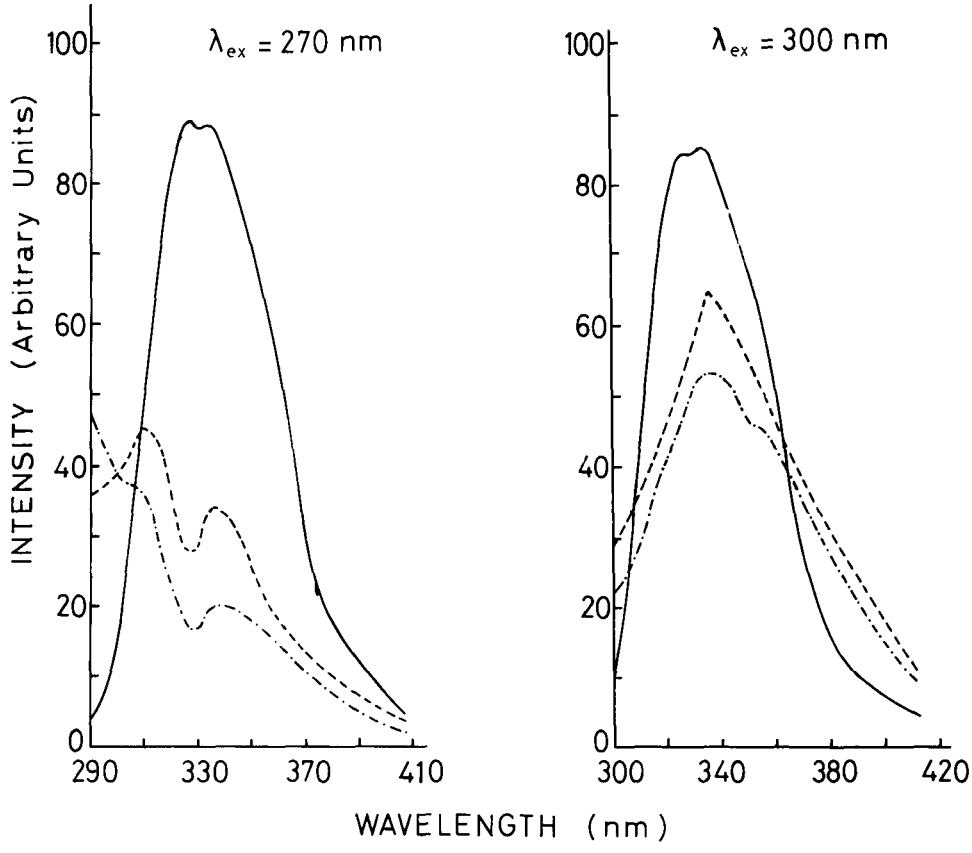


Fig. 2. Corrected fluorescence spectra of tyrosinase in soluble enzyme form (——), immobilized enzyme through primary amino groups (--), and immobilized enzyme through its carbohydrate moiety (- · -). Protein concentration: 16 μg/mL in 0.1*M* phosphate buffer, pH 7.0, and 20°C. Enzyme activity: SE, 16 U/mg; IME, 2.5 U/mg; $IME_c$, 1.0 U/mg.

Independently of the excitation wavelength used, denaturation was also characterized by an increase in half-band width of the spectra of enzyme and proenzyme the broadening being higher for the former.

This difference in half-band width between $SP_D$ and $SE_D$ could be ascribed to a higher unfolding degree of the enzyme versus the proenzyme when subjected to the same denaturing conditions. In both situations, the activity was lost, but owing to the more unfolded conformation of the native soluble enzyme, it seemed that the same kind of conformational relationship was maintained upon denaturing conditions.

### *Fluorescence Quenching of Soluble Tyrosinase*

Figures 4 and 5 show the Stern-Volmer plots for acrylamide and iodide quenching of both soluble proenzyme and enzyme, respectively, in different conformational states (native, denatured, and apoprotein). The experimental data produced straight lines, indicating a low hetero-

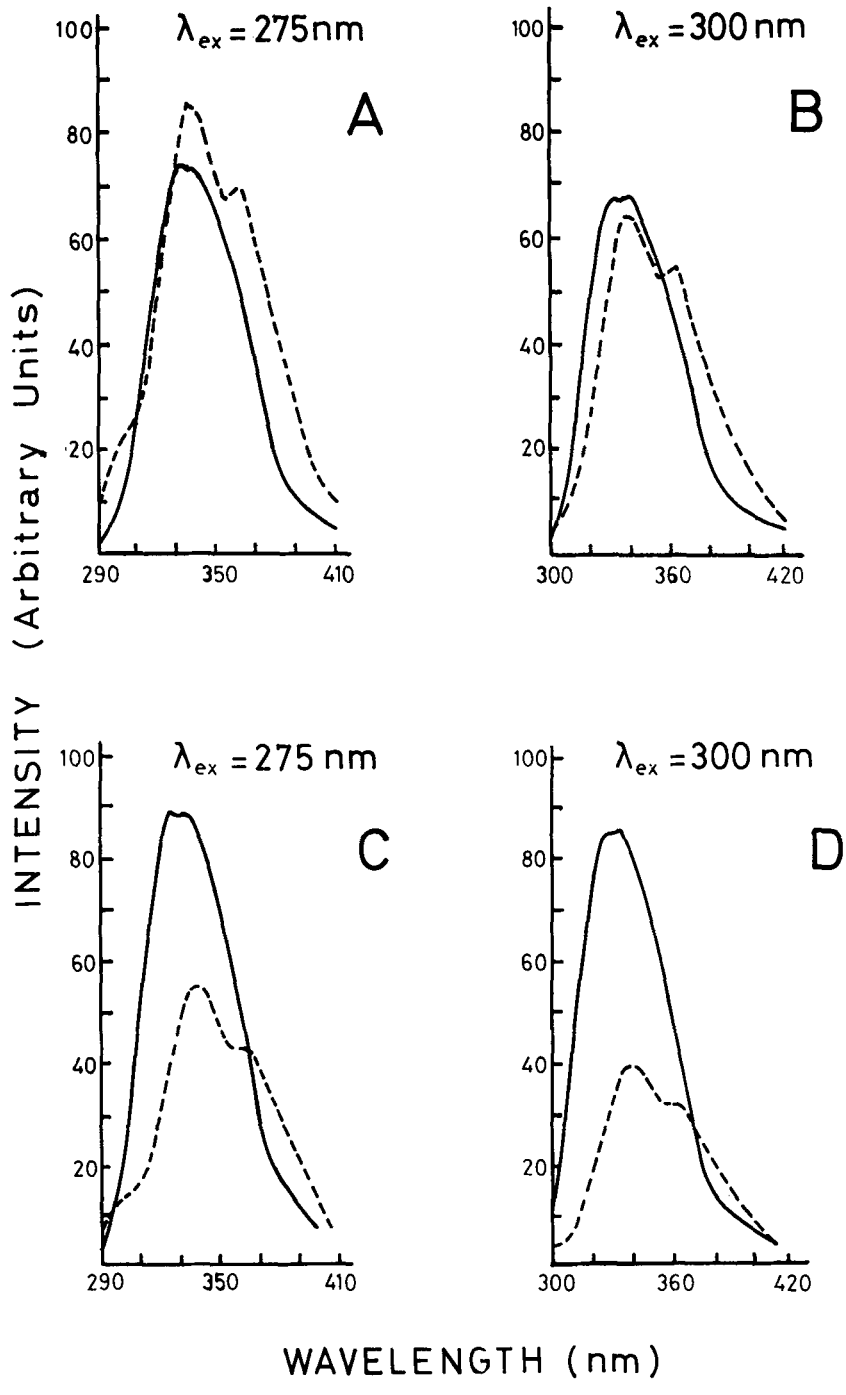


Fig. 3. Corrected fluorescence spectra of soluble tyrosinase in native (solid line) and 3*M* guanidinium chloride denatured (dotted line) forms: A and B, proenzyme; C and D enzyme. Protein concentration in all figures: 26 μg/mL in 0.1*M* phosphate buffer, pH 7.0, and 20°C. Enzyme activity: 16 U/mg.

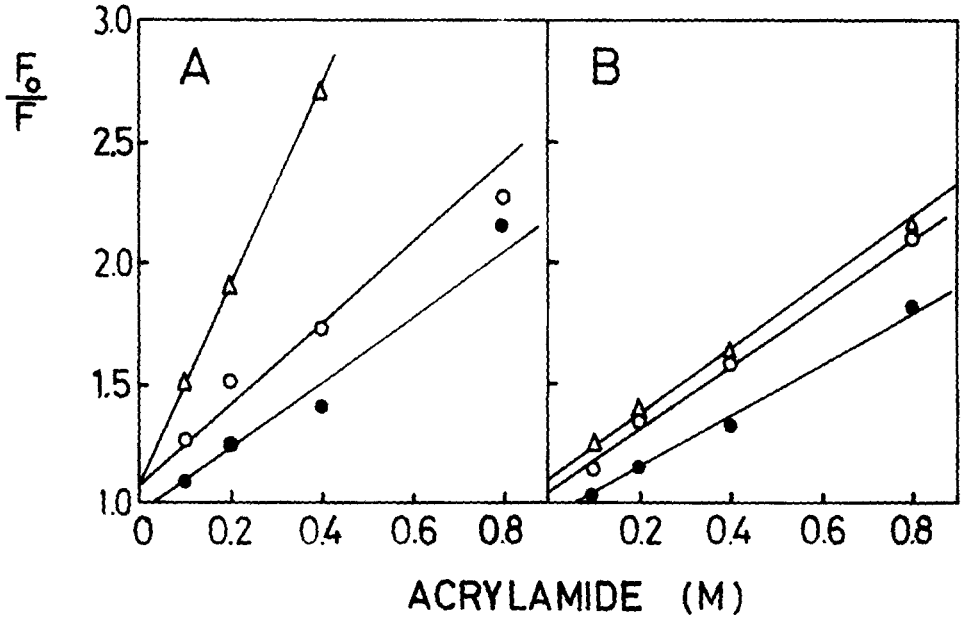


Fig. 4. Stern-Volmer plots obtained for acrylamide quenching of intrinsic tyrosinase fluorescence in soluble proenzyme (A) and enzyme (B) forms. Native (●), denatured (△), and apo-enzyme (○) forms all were tested at the same protein concentration (30–35 μg/mL) in 0.1*M* phosphate buffer, pH 7.0, and 20°C.

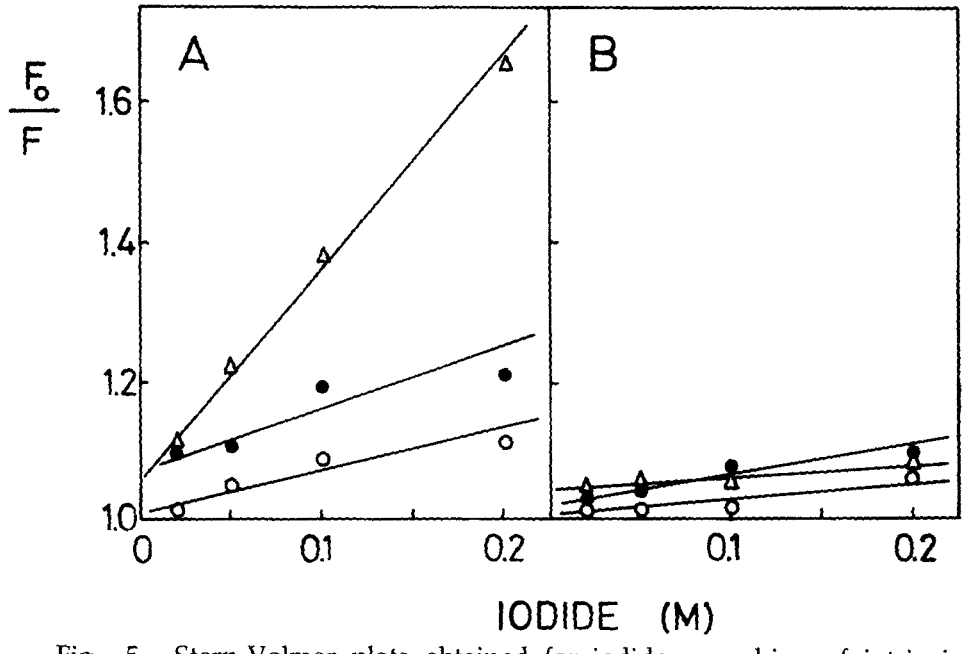


Fig. 5. Stern-Volmer plots obtained for iodide quenching of intrinsic tyrosinase fluorescence in soluble proenzyme (A) and enzyme (B) forms. Native (●), denatured (△), and apo-enzyme (○) forms all were tested at the same protein concentration (30 μg/mL) in 0.1*M* phosphate buffer pH 7.0 and 20°C.

geneity of tryptophyl emission caused by differences in the microenvironment that surrounds these residues in the protein (quenching constants differing by a factor lower than four) (*11*). The quenching constants ($K_Q$) are given in Table 2.

Examination of this table shows a high difference between the quenching parameters obtained for acrylamide and iodide acting either on native proenzyme or enzyme. That result claims for an electronegative microenvironment of tryptophyl residues that avoids fluorescence quenching by iodide.

Acrylamide and iodide quenching seemed to be more easily accomplished in SP than in SE. Then, tryptophyl residues had to be changed to a less accessible position upon protyrosinase activation. These results, and the spectra shown in Fig. 1, suggest that activation involves an unfolding of the protein chain in the sense of an increase in the exposition degree of tyrosyl residues, and a higher shielding of tryptophyl residues.

Treatment with 3*M* guanidinium chloride gave an easily quencheable SP, but had lesser effects on SE.

Copper extraction made tryptophyl residues of soluble both proenzyme and enzyme more quencheable by acrylamide. Thus, copper atoms should be located near tryptophyl residues. This was confirmed when iodide was used as a quencher; copper withdrawal led to an electronegative microenvironment around tryptophyl residues, thus avoiding the iodide quenching. This situation resulted in lower quenching constants for apotyrosinase than for the native protein (Table 2).

### *Fluorescence Quenching of Immobilized Derivatives*

Figures 6 and 7 show the modified Stern-Volmer plots for acrylamide and iodide quenching (respectively) of IME and $IME_c$, in the "native," denatured, and apo forms. Modified Stern-Volmer plots were used since the experimental data did not fit the direct classical Stern-Volmer equa-

TABLE 2
Quenching Constants Obtained for the Collisonal Quenching of Tyrosinase as Calculated from Stern-Volmer Plots (for Soluble Tyrosinase) and by Modified Stern-Volmer Plots (for the Immobilized Derivatives)

| | Quenching constants, $(K_Q)_{eff}$, $M^{-1}$ | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Acrylamide | | | | Iodide | | | |
| Enzyme form | SP | SE | IME | $IME_c$ | SP | SE | IME | $IME_c$ |
| Native | 1.32 | 1.03 | 0.31 | 0.63 | 1.05 | 0.51 | 0.68 | 4.46 |
| Denatured | 4.20 | 1.54 | 0.60 | 1.50 | 3.22 | 0.17 | 2.12 | 1.64 |
| Apo-tyrosinase | 1.48 | 1.37 | 0.82 | 0.35 | 0.62 | 0.28 | 0.50 | 0.73 |

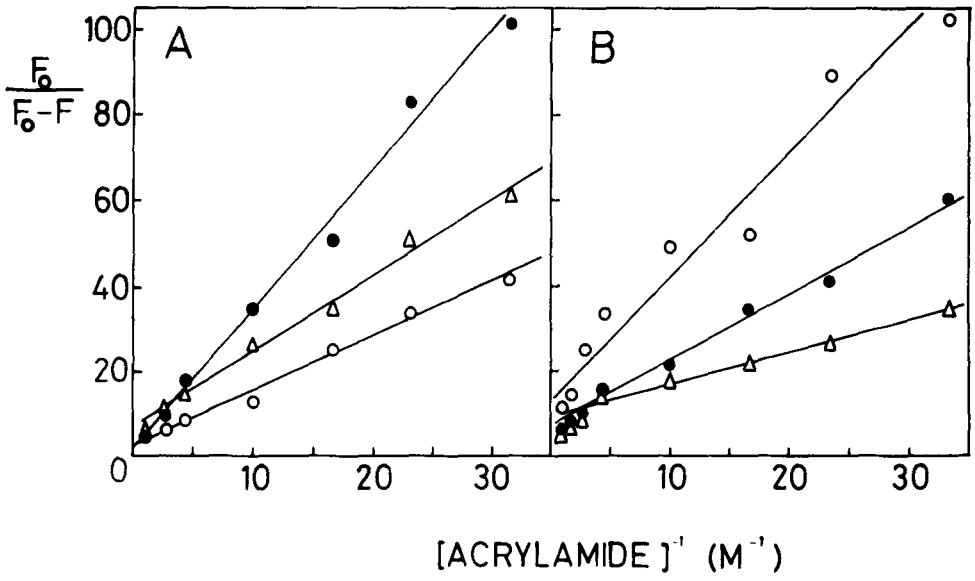


Fig. 6. Modified Stern-Volmer plots for the acrylamide quenching of tryptophyl fluorescence ($\lambda_{ex}$ 300 nm) of tyrosinase immobilized through primary amino groups (A), and through the carbohydrate moiety (B). Native (●), denatured (△), and apo-enzyme (○) forms of the immobilized derivatives were used at the same protein concentration (16 μg/mL).

tion. Then, the emission has to be heterogeneous, and the fluorescence of certain tryptophyl residues is selectively quenched before others (*20*).

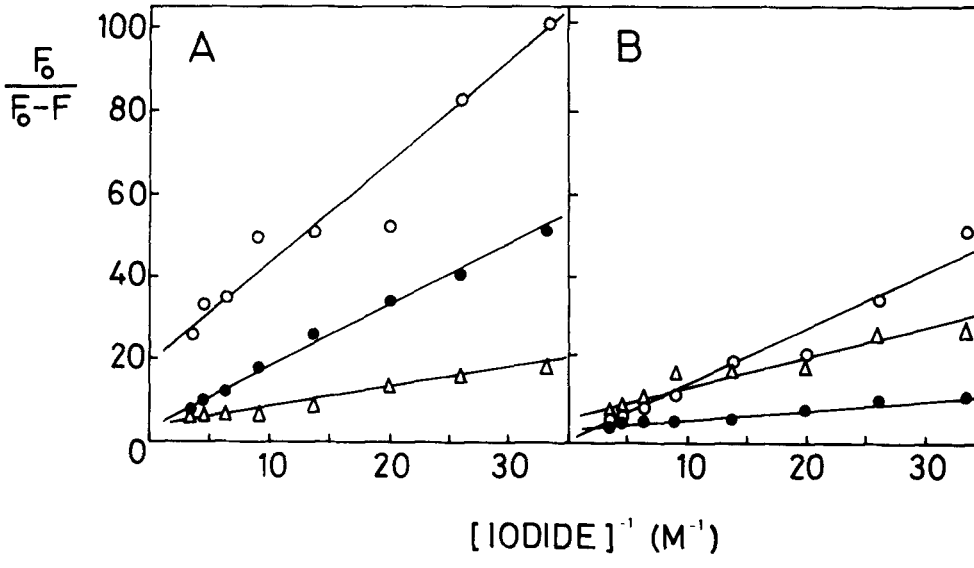


Fig. 7. Modified Stern-Volmer plots for the iodide quenching of tryptophyl fluorescence ($\lambda_{ex}$ 300 nm) of tyrosinase immobilized through primary amino groups (A), and through the carbohydrate moiety (B). Native (●), denatured (△), and apo-enzyme (○) forms of the immobilized derivatives were used at the same protein concentation (15 μg/mL).

Table 2 summarizes the corresponding collisonal quenching constants, showing that tryptophyl residues were less accessible to acrylamide in the immobilized derivative than in soluble tyrosinase. This can be caused by the steric hindrance of the support. It is widely known that immobilization of enzymes renders those areas of the protein that are in close contact with the matrix less accessible to reagents, being that inaccessibility higher as the reagent becomes larger in size (*21*). Although quenching of immobilized proteins has not yet been studied, steric hindrance to accessibility of quenchers to tryptophan has been observed by comparing quenching constants calculated for soluble tryptophan, for tryptophan included in SDS micelles, or for tryptophan as a part of a protein (*22*).

The difference in quenching constants for acrylamide shows that tryptophyl residues in $IME_c$ have to be less occluded than in IME. Two reasons account for this observation: (1) the carbohydrate moiety of tyrosinase is polar enough to be located on the surface of the protein projecting out of the protein, while amino groups, although charged, belong to the protein structure itself; (2) a spacer arm was used when the latter was immobilized through its carbohydrate moiety.

Although the number of linkages between support and protein in each situation was not known, it is reasonable to assume a higher number of bonds between matrix and enzyme for IME than for $IME_c$, since the higher amount of lysine (5.5%) versus carbohydrate (1%) residues for tyrosinase. In fact, although both derivatives became inactive at 6*M* guanidinium-HCl, the tryptophyl residues of $IME_c$ were more easily quenched than those pertaining to IME, thus pointing to a more unfolded conformational state of $IME_c$ upon denaturation, a consequence of its lowered conformational stabilization caused by the number of linkages formed.

Immobilized derivatives were more easily quenched by iodide than the soluble forms. Because the Sepharose matrix imposes diffusion constraints on the passage of soluble molecules, as shown by acrylamide, the electrostatic charge around the protein must obviously be positive to account for these results (*10,23*). In addition to the electrical charge from the support, the enzyme was enriched in amino groups prior to its immobilization through the carbohydrate moiety, and that might well explain the high difference in iodide quenchability between IME and $IME_c$.

Immobilization of tyrosinase through its primary amino groups has to produce a conformational change in which tryptophyl residues attain a localization nearer to the copper atoms of the active center, as evidenced by the effect of copper extraction on tryptophyl quenchability by acrylamide or iodide; the amino acid residues become more accessible to acrylamide, but iodide encountered difficulties to approximate since the electronegative microenvironment resulting from copper removal.

Thus, as seen by fluorescence measurements and quenching studies, activation of tyrosinase is accompanied by a conformational change in which the tryptophyl residues move to the active site, since both tryp-

sin treatment and immobilization produced the same effect and both kinds of treatments are known to activate the proenzyme.

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