Subunit interactions in tyrosinase from frog epidermis in immobilized enzyme systems

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1. Frog epidermis tyrosinase was coupled to Sepharose activated with low concentrations of CNBr. The tetrameric form of the enzyme was linked to the matrix via its subunits. Dissociation of the bound active enzyme with guanidinium chloride yielded an active immobilized dimeric derivative. 2. Immobilized dimeric derivative was able to interact with soluble subunits formed transiently during renaturation. An 85% recovery of the native dopa oxidase specific activity was achieved after hybridization. 3. Fluorescence spectra of different immobilized derivatives suggested that tryptophan residues were involved in the interactions between tyrosinase subunits. 4. It is suggested that the activation of the subunits of tyrosinase involves a conformational change towards a more unfolded state, which favours a reassociation to the dimeric active state.

Tyrosinase (EC 1.14.18.1) is the only known enzyme participating in the process of melanogenesis. It catalyses the hydroxylation of tyrosine to dopa and the oxidation of dopa to dopaquinone. Atmospheric oxygen is the oxidant in both processes. The two types of activity are referred to as hydroxylase and oxidase respectively. Amphibian epidermal tyrosinase is extracted in the proenzyme form and is easily activated by proteinases (McGuire, 1970), light (Mikkelsen *et al.*, 1975), heat (Chen, 1975) and immobilization on a solid support (Iborra *et al.*, 1979). Frog epidermis tyrosinase of *Rana pipiens* seems to have a tetrameric structure in both active and inactive forms of the enzyme (Barisas & McGuire, 1974; Mikkelsen & Triplett, 1975). From the results of cross-linking experiments, we have established that tyrosinase from frog epidermis of *Rana esculenta ridibunda* is formed from four identical or similar subunits, and the existence of an association–dissociation equilibrium between them (J. L. Iborra, J. A. Ferragut & J. A. Lozano, unpublished work). Equilibrium between monomeric, dimeric and tetrameric forms was dependent on protein concentration and was different for proenzyme and enzyme. The mushroom tyrosinase equilibrium has also been previously studied (Fling *et al.*, 1963; Jolley *et al.*, 1969). Nevertheless, the regulatory and/or catalytic func-

tion of the association–dissociation phenomenon has not been yet determined.

One approach to understanding the function of quaternary structures in enzymes is to study the properties of single subunits and to compare them with those of the native oligomer. Chan (1970) introduced the use of immobilization techniques in order to study possible activity of the isolated subunits under conditions in which reassociation was prevented, and subsequent reports demonstrated the existence of active subunits bound to a solid matrix (Chan & Mawer, 1972; Chan *et al.*, 1973; Grazi *et al.*, 1973; Green & Toms, 1973; Chan, 1973, 1974; Fell & White, 1975; Bickerstaff & Price, 1976; Bruch *et al.*, 1976; Chan & Mosbach, 1976; Carvajal *et al.*, 1977; Gurne *et al.*, 1977; Batlle *et al.*, 1978; Chantler & Gratzer, 1978; Kochetov & Solovieva, 1978; Andersson & Mosbach, 1979; Arrio-Dupont & Coulet, 1979; McCracken & Meighen, 1979). The use of immobilized enzyme as a tool for evaluating subunit interactions has been reviewed (Chan, 1976a,b; Bickerstaff, 1980). This technique may be applied to the investigation of hybridization between bound and nascent soluble subunits (Feldmann *et al.*, 1972, 1976) and also to protein purification by subunit-exchange chromatography (Gurne *et al.*, 1977; Rossi-Fanelli *et al.*, 1978; Van Etten & Waheed, 1980).

The present work attempts to study the catalytic function of tyrosinase subunits linked covalently to CNBr-activated Sepharose and their interactions

Abbreviations used: dopa, 3,4-dihydroxyphenylalanine; SDS, sodium dodecyl sulphate.

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with other tyrosinase subunits added in solution. We try to relate these catalytic activities to the association-dissociation equilibrium.

Experimental

Materials

Frogs (*Rana esculenta ridibunda*) were obtained from local suppliers. Trypsin (EC 3.4.21.4) was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Sepharose 4B, CM-Sephadex and phenyl-Sepharose were obtained from Pharmacia (Uppsala, Sweden). CNBr, dopa and all the other reagents were analytical grade from E. Merck (Darmstadt, Germany).

Preparation of soluble enzyme

Frog epidermis tyrosinase was prepared as proenzyme as described by Iborra *et al.* (1977) and was purified by successive chromatography on CM-Sephadex and phenyl-Sepharose, as follows. About 0.5 g of dry CM-Sephadex C-50 was swollen in 50 mM-sodium phosphate buffer, pH 7.0, and poured into a glass filter. A 200 ml volume of proenzyme extract in the above buffer was added to gel in small portions (10–15 ml), with stirring. After 5 min no oxidase activity was detected in the extract. Tyrosinase was desorbed with 0.1 M-sodium phosphate buffer, pH 7.0, and 10–15 ml fractions were collected until no protein was detected. These fractions were used for phenyl-Sepharose chromatography. The hydrophobic-interaction chromatography was performed in the following way. Phenyl-Sepharose CL-4B (5 ml) was placed in a Quickfit 10/20 column and washed with 0.5 M-sodium phosphate buffer, pH 7.0. The proenzyme solution obtained in the CM-Sephadex step (200 ml), previously adjusted to 0.5 M-sodium phosphate buffer, was passed through the column at a rate of 24 ml/h, and washed with the same buffer until no protein was detected in the eluates. Then 0.1 M-sodium phosphate buffer, pH 7.0, was used, and 3 ml fractions were collected. The purified proenzyme solution was concentrated by ultrafiltration on an Amicon CF-25 filter. The concentrated solution was submitted to SDS/polyacrylamide-gel electrophoresis (Weber & Osborn, 1969). Activation of the proenzyme was accomplished by using a trypsin-Sepharose column, and enzyme activity was determined by the formation of dopachrome as previously described (Iborra *et al.*, 1977). Protein was determined by a modified Lowry method (Hartree, 1972), with bovine serum albumin as standard.

Preparation of tyrosinase-Sepharose

Sepharose 4B was activated with CNBr as described by Axen & Ernback (1971). A 0.05–0.5 mg quantity of CNBr was used to activate 1 ml

of the packed gel for attachment of the enzyme to the matrix via a single subunit. The activated groups of support react with primary amino groups of the enzyme to form an isourea linkage. Then a 200 mg amount of CNBr was used when immobilization through multiple unions was made. The procedure for immobilization of tyrosinase as proenzyme and enzyme forms on CNBr-activated Sepharose has been published elsewhere (Iborra *et al.*, 1977, 1979). The gels obtained were suspended in 0.1 M-sodium phosphate buffer, pH 7.0, for later utilization. The quantity of tyrosinase coupled to the support was evaluated by Hartree's (1972) procedure by measuring the protein concentration of the tyrosinase solution before and after the coupling process.

Denaturation of tyrosinase

Denaturation of different derivatives of tyrosinase was performed by treating 0.5 ml of immobilized tyrosinase derivatives or 0.1 ml of soluble enzyme, for 10 min at 25°C, with a 0.01–6.0 M range of guanidinium chloride concentration in 0.1 M-sodium phosphate buffer, pH 7.0. Enzymic activity was checked at different times for each guanidinium chloride concentration. Renaturation of guanidinium chloride-pretreated soluble enzyme was performed by dialysis with 0.1 M-sodium phosphate buffer, pH 7.0, or by using 1:200 dilution in the same buffer solution. This last method was employed for renaturation of the previously denatured immobilized preparations.

Preparation of subunit tyrosinase-Sepharose

The subunit tyrosinase-Sepharose was prepared as follows. A 1 ml volume of tyrosinase-Sepharose linked via a single subunit was packed in a column (1 cm internal diam.) and washed with 20 ml of 0.1 M-sodium phosphate buffer, pH 7.0. Four more suspension-filtration cycles were performed at room temperature with a similar buffer but containing 3 M-guanidinium chloride in order to remove the subunits not attached to the support. Subunit tyrosinase-Sepharose renaturation was achieved by washing the insoluble derivative with 10 ml of 10 mM-sodium phosphate buffer, pH 7.0. The same operation was repeated seven times.

The amount of subunit coupled to the support was determined by SDS/polyacrylamide-gel electrophoresis. The protein eluted after the subunit tyrosinase-Sepharose denaturation procedure was concentrated to 1 ml by ultrafiltration in an Amicon cone with a CF-25 membrane. A 50 µl volume of this solution was subjected to SDS/polyacrylamide-gel electrophoresis in gels containing 5% acrylamide as described by Weber & Osborn (1969). Protein bands were stained with Coomassie Blue R-250 in 10% acetic acid. Stained protein bands in gels were measured at 600 nm in a Transdyne densitometer and

evaluated by comparison with a calibration curve obtained with known amounts of purified tyrosinase ranging from 4 to 22 μg of protein (Fig. 1). In this way it was possible to know the amount of protein not coupled covalently to the Sepharose as established by Chantler & Gratzer (1978). The difference between protein quantity before (1.2 mg) and after the coupling process (0.7 mg) was the protein covalently bound to the support (0.5 mg). The percentage of immobilized enzyme was 40% of the applied enzyme.

Interaction of Sepharose derivatives with added soluble subunits

Subunit tyrosinase-Sepharose was reassociated with other soluble enzyme subunits in order to obtain a renatured tyrosinase-Sepharose by the following procedure (Chan, 1976a). Subunit tyrosinase-Sepharose (3 ml packed volume) was suspended in 200 ml of 0.1 M-sodium phosphate buffer, pH 7.0, and was gently stirred at room temperature with a magnetic stirrer.

A 50 μl volume of a solution containing 20 μg of previously denatured and dissociated tyrosinase in guanidinium chloride was added to the suspension. At 10 min intervals 60 more additions were made. After the last addition the mixture was left for 1 h in order to obtain a good interaction. Then the gel was washed with 0.1 M-sodium phosphate buffer, pH 7.0, and filtered thorough Amicon filters. This cycle was repeated several times, and a final volume of 3 ml was obtained. Samples were assayed for activity.

Fig. 1. Linearity of the calibration of the area under the peak in the stained densitometer trace and the tyrosinase applied to electrophoretic gels

The areas under the peak were measured by planimetry. The experimental points were fitted to a straight line by least-mean-square analysis ($r = 0.994$). The concentration of tyrosinase eluted after the subunit tyrosinase-Sepharose denaturation procedure was determined by interpolation in the calibration curve (arrow). The running buffer was SDS/Tris/glycine, pH 8.6. For experimental details see the text.

Assays of activity of immobilized derivatives

The activity of different immobilized derivatives was measured by continuous polarographic analysis of the oxygen consumption in a stirred batch reactor (Mattiason & Mosbach, 1976).

A Clark electrode connected to an oxygen monitor (Yellow Springs YSI 53) with thermostatically controlled cells at $25.00 \pm 0.01^\circ\text{C}$ was used for this purpose. After calibration of the electrode response, 2.5 ml of L-dopa (2 mg/ml) or 2 mM-L-tyrosine in 0.1 M-sodium phosphate buffer, pH 7.0, was placed in the cell, and the solution was saturated with air at 26°C . After the electrode current became constant, 0.5 ml of swollen immobilized-derivative gel was added. The mixture was stirred and the course of oxygen consumption with time was registered. The difference between the initial value and the final plateau corresponds to the amount of substrate oxidized.

The activity of immobilized derivatives was related to that of the soluble enzyme by means of a calibration curve of electrode response against known soluble-enzyme concentrations (expressed as activity units). Whenever possible, after the required recorder tracing of electrode-response change had been obtained, the assay-mixture supernatant was checked for soluble enzyme activity. Enzyme derivatives obtained in this way contained no detectable soluble enzyme activity.

When proenzyme was used for immobilization studies, a previous activation of the immobilized proenzyme was necessary for activity assays. The activation was performed by addition of 10 μl of trypsin solution (1 mg/ml) to 0.5 ml of swollen gel, and the mixture was incubated for 5 min at 37°C .

Fluorescence

Fluorescence measurements were made in a Perkin-Elmer model 24 spectrofluorimeter, provided with xenon lamp. Light-excitation was performed at 280 and 290 nm in order to evaluate the contribution of tyrosine and tryptophan residues respectively (Shifrin & Parrot, 1974). Samples of gel (50 μl) or soluble enzyme (50 μl) were suspended or dissolved in 0.1 M-sodium phosphate buffer in a 1 cm-light-path cuvette, a 3 ml final assay volume being used. All spectra were relative and uncorrected. Sepharose 4B and phosphate solution alone showed no fluorescence, but only the normal scattering peak at 280 or 290 nm.

Results

Enzyme purity

Table 1 shows the results obtained in the purification of frog epidermis tyrosinase. As much as 85% of the enzyme present in the cell extract was

Table 1. *Purification of frog epidermis tyrosinase*
For experimental details see the text.

Stage of preparation	Total activity (units)	Specific activity (units/mg)	Recovery of activity (%)	Purification factor (fold)
Cell extract	912	7.1	100	3.0
CM-Sephadex	840	36.2	92	15.3
Phenyl-Sepharose	798	95.0	86.6	39.8

Table 2. *Dopa oxidase activity and protein content of tyrosinase derivatives*

For experimental details see the text. The protein-content values are expressed as $\mu\text{g/ml}$ of packed gel. The specific activity of native soluble tyrosinase was 29.3 units/mg under the conditions employed for assay.

Immobilized derivative	Activity (units/ml)	Recovery of activity (%)	Protein content ($\mu\text{g/ml}$)	Recovery of protein (%)	Specific activity (units/mg)
Tyrosinase-Sepharose	0.72	100	500	100	14.4
Subunit tyrosinase-Sepharose	0.35	48.6	240	48	14.5
Renatured tyrosinase-Sepharose	0.60	85.0	—	—	—

recovered in the phenyl-Sepharose effluent. The SDS/polyacrylamide-gel electrophoresis pattern of a purified proenzyme sample is illustrated in Fig. 2. Densitometry of stained gel indicated that almost all of the stained protein (95%) was present in a single peak.

Activity and protein content of immobilized enzyme derivatives

The preparation of tyrosinase bound to Sepharose activated with low amount of CNBr (2 mg/ml of Sepharose) gave a derivative that contained significant bound-enzyme activity (Table 2). At this low amount of CNBr it could be expected that almost all of the bound enzyme molecules would be covalently linked to the polysaccharide matrix subunits (Chan, 1970, 1976b).

This immobilized enzyme preparation was incubated with guanidinium chloride to dissociate the tyrosinase-Sepharose derivative into subunits, and to characterize the properties of bound subunits. Fig. 3 (inset) shows the effect of different concentrations of guanidinium chloride on the activity of tyrosinase-Sepharose; the results indicate that tyrosinase-Sepharose is completely denatured in 40 mM-guanidinium chloride. Fig. 3 also shows that inhibition of soluble enzyme and tyrosinase immobilized through multiple interaction with guanidinium chloride rapidly increased with increasing guanidinium chloride concentrations; soluble enzyme needs a guanidinium chloride concentration of about 2 M for 100% inhibition, whereas immobilized enzyme became fully denatured in 5.6 M-guanidinium chloride. This difference would mean a stabilizing effect of the matrix on the immobilized

Fig. 2. *Analytical electrophoresis of tyrosinase proenzyme on polyacrylamide gels*

A sample of the proenzyme preparation from the phenyl-Sepharose step was submitted to polyacrylamide-gel (5%) electrophoresis in the presence of SDS. The amount of protein was 20 μg . The running buffer was SDS/Tris/glycine, pH 8.6. Protein bands were stained with Coomassie Blue. For experimental details see the text.

enzyme due to support rigidity. The inhibition profiles for soluble and immobilized enzymes also suggest the existence of intermediate states during denaturation by guanidinium chloride.

The tyrosinase-Sepharose derivative was treated

Fig. 3. Inactivation of tyrosinase derivatives by guanidinium chloride

The conditions of denaturation are described in the Experimental section. ●, Activity of soluble enzyme; ○, activity of enzyme immobilized through multiple union; △ (inset), activity of tyrosinase-Sephadex.

with 3M-guanidinium chloride and washed repeatedly with pH 7.0 buffer to remove any denatured polypeptides that might be non-covalently bound to the matrix. After that treatment this immobilized enzyme preparation would be dissociated into subunits. The product was washed with phosphate buffer to allow the covalently bound enzyme subunit to refold. This subunit tyrosinase-Sephadex derivative showed dopa oxidase activity that was nearly 50% of that of tyrosinase-Sephadex (Table 2). The protein content of subunit tyrosinase-Sephadex was about half the amount of that of tyrosinase-Sephadex, as determined by interpolation in the calibration curve described in the Experimental section. These findings indicate that two subunits are involved in the binding of tyrosinase to the support, and it is noteworthy that the specific activity of the subunit tyrosinase-Sephadex was close to that of the tyrosinase-Sephadex. The subunit tyrosinase-Sephadex showed no detectable dopa oxidase

activity at 10mM-guanidinium chloride, whereas tyrosinase-Sephadex was 30% active at this concentration of denaturant. This result is consistent with the presence of spatially isolated subunits (Chan, 1976a).

On the other hand, a 50% renaturation was obtained from denatured soluble enzyme by the dialysis method in the course of 1 h at different protein concentrations (20–200 µg/ml) or by the dilution method. For enzyme immobilized through multiple union incubated with 5.6M-guanidinium chloride only 20% renaturation was achieved. A higher renaturation percentage for subunit tyrosinase-Sephadex (45%) than for enzyme immobilized through multiple union (20%) also confirmed the existence of active immobilized subunits. The obtaining of plateaus showing 50% activity during the denaturation process for soluble and for immobilized enzyme (Fig. 3) and the maximum activity achieved (50% of that of the native enzyme) for renaturation of the soluble enzyme represents 50% irreversible denaturation.

When tyrosine hydroxylase activity of enzyme immobilized through multiple union was assayed, the oxygen consumption was 3000-fold greater than when dopa was used as substrate. The enzyme immobilized through multiple union needed a 7.6M-guanidinium chloride concentration to attain 55% inhibition of hydroxylase activity. On the other hand tyrosine hydroxylase activity of the tyrosinase-Sephadex derivative was inhibited fully in 6.4M-guanidinium chloride, whereas as little as 40mM-guanidinium chloride was necessary to obtain a 100% denaturation of dopa oxidase activity.

Effect of CNBr on the coupling reaction and on the probability of interaction between subunits

In the preparation of tyrosinase immobilized through a single subunit we found that there was a relationship between the amount of CNBr used to activate the gel and the ratio of activities between subunit tyrosinase-Sephadex and tyrosinase-Sephadex (Fig. 4). At amounts of CNBr ranging between 1 and 5 mg of CNBr/ml of packed gel a 50% relative activity of subunit tyrosinase-Sephadex to that of tyrosinase-Sephadex was obtained. We do not know exactly the number of sites of attachment of the tyrosinase to the gel; however, these results also suggest that dimeric species are involved in the union to the support.

The low catalytic activity observed in the immobilized subunit preparation at low extent of gel activation (0.5 mg of CNBr/ml of gel) can be related to an anomalous renaturation of subunit tyrosinase-Sephadex, an adsorbed enzymic activity on gel interstices or a threshold activity of immobilized dimers, as obtained by Feldmann *et al.* (1976) with glycogen phosphorylase.

Table 3. Calculations of fractions of subunits with neighbour nearer than $r = 0.6 \text{ nm}$ ($6 \text{ \AA}$) for tyrosinase ϕ_1 gives the fraction of molecules with one neighbour within a distance of $r = 0.6 \text{ nm}$ ($6 \text{ \AA}$) (Lozano *et al.*, 1975), $\bar{r}$ is the radius in 0.1 nm ($\text{\AA}$) of a sphere that contains on the average one subunit ($\bar{r} = 7.36 \text{ C}^{-1}$, where C is the effective molar concentration of tyrosinase subunits) and were calculated as expressed by Green & Toms (1973).

CNBr (mg/ml of gel)	CNBr (mM)	$\bar{r}$		$r/\bar{r}$	ϕ_1
		(nm)	($\text{\AA}$)		
0.5	0.22	1.219	12.19	0.49	0.12
1.5	0.65	0.847	8.47	0.70	0.30
2.5	1.09	0.715	7.15	0.84	0.45
4.0	1.75	0.610	6.10	0.98	0.62
5.0	2.11	0.575	5.75	1.04	0.65

Fig. 4. Dependence of relative activity of subunit tyrosinase-Sepharose to tyrosinase-Sepharose with the extent of activation by CNBr

Tyrosinase-Sepharose and subunit tyrosinase-Sepharose derivatives were obtained by using a quantity of 0.5 to 5.0 mg of CNBr to activate 1 ml of Sepharose, as described in the Experimental section. The activity of each derivative, expressed in units/ml of packed gel, was measured by polarographic analysis of the oxygen consumption, as indicated in the Experimental section.

The estimation of the fraction of multiple-linked tyrosinase molecules present in the parent preparation of coupled tetramer as a function of the amount of CNBr used in the coupling reaction was made as established by Green & Toms (1973). The calculations of fractions of subunit with a neighbour nearer than 0.6 nm ($6 \text{ \AA}$) (Stokes radius of frog epidermis tyrosinase calculated by gel filtration) (Lozano *et al.*, 1975) are expressed in Table 3 for a

Fig. 5. Comparison of fluorescence spectra of soluble proenzyme and active tyrosinase

Curve A, proenzyme, 0.3 mg/ml; curve B, enzyme, 0.2 mg/ml. Spectra were obtained in 0.1 M-phosphate buffer, pH 7.0, as described in the Experimental section. Excitation wavelength was 280 nm.

CNBr range between 0.5 and 5 mg/ml of packed gel. A 60% probability of an immobilized subunit having another one in its immediate vicinity is obtained at 4 mg of CNBr/ml of Sepharose, which indicated that in this condition most of the tyrosinase molecules were bound to the support by more than one subunit.

Interactions of subunit tyrosinase-Sepharose with subunits in solution

In order to test whether the subunit tyrosinase-Sepharose would pick up subunits from a

solution, small volumes of the dissociated soluble enzyme were added to a much larger volume of phosphate buffer containing the subunit tyrosinase-Sepharose, in accordance with the technique developed by Chan (1973). Data in Table 2 show that immobilized subunits are able to reassociate with the native subunits generated *in situ* to yield a product (renatured tyrosinase-Sepharose) resembling the original bound enzyme and showing 85% of the tyrosinase-Sepharose activity. These observations also suggest that immobilized subunits show a specific affinity towards other native subunits and that they had a dopa oxidase activity similar to that of the tyrosinase-Sepharose preparation from which they were derived.

Conformational transitions of soluble and immobilized tyrosinase derivatives

Fluorescence spectra of proenzyme and enzyme tyrosinase in solution are shown in Fig. 5. The

results, analysed in terms of either fluorescence intensity or the wavelength of maximum emission, indicated the occurrence of a conformational change in the transition from proenzyme to enzyme. The fluorescence peak was blue-shifted about 10nm (from 337nm to 328nm), and the fluorescence intensity increased about 2-fold as a consequence of the change in the environment of hydrophobic residues as a result of the activation.

Spectra obtained for the immobilized enzyme derivatives and the proenzyme ones are shown in Figs. 6 and 7 respectively. As observed, the fluorescence peak was also blue-shifted when both proenzyme and enzyme were immobilized. This could be caused by the conformational changes produced on the protein by its union to the support. Furthermore, the fluorescence spectra of tyrosinase conjugated with Sepharose did not resemble in shape those obtained for the free protein. A new

Fig. 6. Fluorescence spectra of immobilized active tyrosinase derivatives

Spectra were obtained after suspension of a volume of packed gel in 0.1 M-phosphate buffer, pH 7.0, to a final volume of 3 ml. Curve A, immobilized enzyme through multiple union, gel volume 50 μ l, protein concentration 120 μ g/ml of gel; curve B, tyrosinase-Sepharose, gel volume 5.0 μ l, protein concentration 500 μ g/ml of gel; curve C, subunit tyrosinase-Sepharose, gel volume 50 μ l, protein concentration 240 μ g/ml of gel. Excitation wavelength was 280 nm.

Fig. 7. Fluorescence spectra of immobilized proenzyme derivatives

Spectra were obtained after suspension of a volume of packed immobilized proenzyme derivative in 0.1 M-phosphate buffer, pH 7.0. Curve A, immobilized proenzyme through multiple union, gel volume 50 μ l, protein concentration 110 μ g/ml of gel; curve B, proenzyme-Sepharose, gel volume 50 μ l, protein concentration 450 μ g/ml of gel; curve C, subunit proenzyme-Sepharose, gel volume 50 μ l, protein concentration 200 μ g/ml of gel. The final volume was 3 ml. Excitation wavelength was 280 nm.

fluorescence peak appeared at 337 nm when subunit tyrosinase–Sephadex was excited at 280 nm (Fig. 6, curve C) and at 350–357 nm if excitation was at 290 nm. The fluorescence intensity of subunit tyrosinase–Sephadex increased 2-fold with respect to tyrosinase–Sephadex. Fig. 7 also shows that the maximum fluorescence peak and the fluorescence intensity of immobilized proenzyme derivatives changed in a similar way to those of active enzyme. These changes obviously arise from the transfer of tryptophan residues from the interior of the protein to a more polar aqueous environment.

Discussion

The results described above lead us to the conclusion that a matrix-bound dimeric tyrosinase is present in the subunit tyrosinase–Sephadex product. This interpretation is based not only on the comparison of the recovery of both protein and catalytic activity but also on the different properties, stability to denaturants and interaction with soluble subunits of the derivatives as compared with native tyrosinase (Chan, 1976a,b).

The usual degree of gel activation cited in the literature normally ranges from 1 to 5 mg of CNBr/ml of wet packed gel in order to minimize the possibility of enzyme attachment to agarose via more than one subunit. However, Nagradova *et al.* (1974) reported the existence of immobilized active dimers of the tetrameric glyceraldehyde 3-phosphate dehydrogenase with the use of 20 mg of CNBr/ml of Sephadex. Batlle *et al.* (1978) used 30–50 mg of CNBr to activate 1 ml of gel and to immobilize the octameric 5-aminolaevulinic dehydratase through two subunits. However, Chan & Mawer (1972), using more than 5 mg of CNBr/ml of packed gel, found evidence for some degree of multipoint attachment of tetrameric fructose biphosphate aldolase. Therefore we believe that the isolation of immobilized monomers or dimers by the coupling of oligomeric proteins to Sephadex activated with a low proportion of CNBr appears also to depend on the nature of the enzyme.

Few physicochemical studies of the immobilized subunits have been conducted. The extremely low protein content in such immobilized derivatives is a handicap when absorption-spectroscopic techniques are used. But, owing to its good sensitivity, the fluorescence method can be applied not only for monitoring structural changes occurring in immobilized enzyme derivatives (Swaisgood *et al.*, 1978) but also for the evaluation of subunit interactions in enzymes. The observed red-shifts of about 20 nm for the emission maximum and the increased fluorescence intensities of subunit tyrosinase–Sephadex derivatives as compared with that of the immobilized oligomeric derivatives represent the

contribution of tryptophan residues in the inter-subunit interactions between tyrosinase dimers. These residues would be exposed to a more polar environment after the dissociation and removal of subunits not covalently bound to the matrix. The increase of fluorescence intensity in the subunit proenzyme–Sephadex as compared with subunit enzyme–Sephadex due to alteration of the environment of the tryptophan residues can be ascribed to a greater distortion of the conformation for the proenzyme than for enzyme when both protein forms are immobilized. This conformational change leads to the possibility that intersubunit contacts would be different in proenzyme relation to the enzyme. Moreover, the quenching percentage produced by Cu^{2+} ions on the tryptophan fluorescence is smaller in the proenzyme than in the enzyme, since the metal ion in the proenzyme is more occluded than in the active tyrosinase (J. L. Iborra, J. A. Ferragut & J. A. Lozano, unpublished work).

Although several studies have demonstrated that frog epidermis tyrosinase is a tetrameric enzyme composed of identical subunits (Barisas & McGuire, 1974; Mikkelsen & Triplett, 1975), none has attempted to assess the possible catalytic activity of subunits. The results reported in the present paper, together with the cross-linking experiments performed to study the determination of quaternary structure of tyrosinase (J. L. Iborra, J. A. Ferragut & J. A. Lozano, unpublished work), tempted us to propose that when tyrosinase is activated by trypsin a change towards a more unfolded conformational state of the enzyme takes place (Iborra *et al.*, 1979), and the formation of dimers, which are the catalytically active species, could be a consequence of this conformational change. It is unknown whether or not the monomer form of enzyme has catalytic activity, but Carmona *et al.* (1980) have claimed that tyrosinase is an auto-associating enzyme under the influence of the substrate tyrosine. The diverse effects of guanidinium chloride on the monophenol oxygenase and diphenol oxygenase activities of immobilized derivatives suggest that tyrosinase from frog epidermis is an enzyme with different kinetic behaviour towards tyrosine and dopa (Pomerantz, 1966).

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