Differential Reactivities of Tyrosine Residues of Proteins to Tyrosinase*

Joseph G. Cory† and Earl Frieden

ABSTRACT: The susceptibility of the tyrosyl groups of the dehydrogenase enzymes, alcohol, glutamic, lactic, and glyceraldehyde 3-phosphate, to oxidation by tyrosinase has been studied. The effect of tyrosinase on aldolase has also been studied. The oxidation of the tyrosyl residues of alcohol dehydrogenase by tyrosinase results in an enzyme species which has only partial activity. Although 80% of the tyrosyl groups are oxidized, only 50% of the enzymic activity is lost. The K_m of the oxidized species is the same as untreated alcohol dehydrogenase. Lactic acid dehydrogenase is oxidized to a small extent with no loss in enzymic activity. Glutamic acid dehydrogenase and glyceraldehyde 3-phosphate dehydrogenase are not substrates. Aldolase, when treated with tyrosinase, loses 82% of its activity. Using tyrosinase, it is possible to investigate the role of the tyrosyl residues in the biological activity of certain proteins and to differentiate between the accessibility of the various tyrosyl groups to the enzyme.

The role of the amino acid side chains in the biological activity of many proteins has been extensively studied. More commonly, so-called amino acid specific reagents have been used to modify the side chains (Keil, 1965; Frankel-Conrat, 1959; Porter, 1953; Herriot, 1947). In most cases, the specific reagents of amino acids have not been specific and the modification of more than one type of amino acid residue has resulted. Ray and Koshland (1961) described a method for distinguishing residues reacting with a reagent at different rates, which permitted the loss of biological activity to be associated with a particular residue or group of residues.

It has been shown in several laboratories (Sizer, 1953; Frieden *et al.*, 1959; Yasunobu *et al.*, 1959; Cory *et al.*, 1962; Lissitzky, 1962) that tyrosinase can catalyze the oxidation of the tyrosyl residues of some intact proteins. Furthermore, it has been shown that the tyrosyl groups of amorphous insulin react at two different rates (Cory and Frieden, 1967). In this communication, the use of tyrosinase as a specific amino acid reagent for tyrosyl groups in certain proteins is reported.

Materials

Mushroom tyrosinase (*o*-diphenol:oxygen oxidoreductase, EC 1.10.3.1) used in these experiments was prepared from fresh mushrooms essentially by the method of Frieden and Ottesen (1959). The tyrosinase preparation was electrophoretically homogenous on cellulose acetate strips in barbital buffer, pH 8.6, 0.075 M. Our preparation was devoid of detectable protease activity. The specific activity was 180–250 units/mg.

A tyrosinase unit as defined by Fling *et al.* (1963) is used throughout this paper. The assay mixture consists of 4.0 mg of (L-dopa)[1] in 0.1 M sodium phosphate, pH 6.0, in a final volume of 5.0 ml. The formation of dopachrome is measured at 475 mμ and 30°. This unit, 1 μmole of substrate changed/min, conforms to the recommendations of the Enzyme Commission of the International Union of Biochemistry.

Yeast alcohol dehydrogenase (two times crystallized, 20 units/mg) and rabbit muscle aldolase (29.4 EU/mg) were purchased from Worthington Biochemical Co. Bovine liver glutamic acid dehydrogenase (A grade, 25 EU/mg) and rabbit muscle glyceraldehyde 3-phosphate dehydrogenase (A grade, 25 EU/mg) were purchased from Calbiochem. Rabbit muscle lactic acid dehydrogenase (two times crystallized, 17 EU/mg) was purchased from Mann Chemical Co.

Methods

A Beckman DU spectrophotometer was used to measure the changes in absorbance of the protein substrates at 280 mμ. A Beckman DK-1 recording spectrophotometer was used to measure the rates of reac-

* From the Department of Chemistry and the Institute of Molecular Biophysics Florida State University, Tallahassee, Florida 32306. *Received August 22, 1966.* Supported in part by Grant HE 08344, U. S. Public Health Service. Paper no. 23 in a series from this laboratory on copper-enzymes and copper in biological systems. The use of equipment and services provided by the Institute of Molecular Biophysics is gratefully acknowledged.

† Present address: Department of Chemistry, University of South Florida, Tampa, Fla.

[1] Abbreviations used: L-dopa, 3,4-dihydroxy-L-phenylalanine; dopachrome, 2-carboxy-2,3-dihydroindole-5,6-quinine; GDH and LDH, glutamic and lactic acid dehydrogenases; NAD, nicotinamide–adenine dinucleotide; ADH, yeast alcohol dehydrogenase.

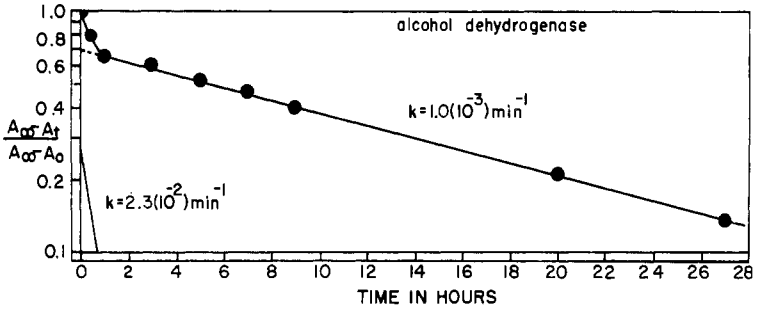

FIGURE 1: First-order plot for the oxidation of yeast alcohol dehydrogenase by tryosinase at 8°. The reaction mixture contained 2 mg of ADH, 0.066 M phosphate buffer, pH 7.5, and 27 units of tyrosinase in a final volume of 3.0 ml. Aliquots were removed and suitable dilutions were made so that the change in absorbance at 280 mμ could be followed as a function of time.

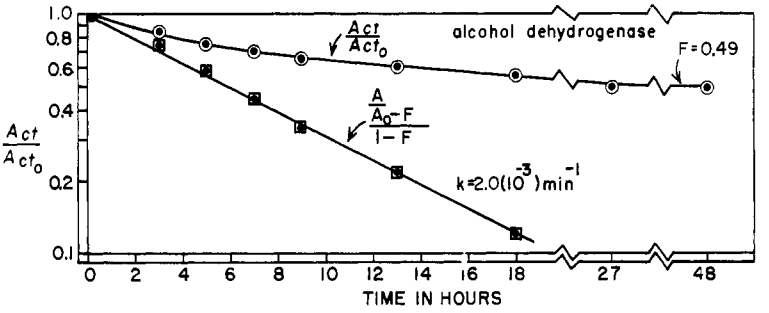

FIGURE 2: A semilog plot for the loss of ADH activity with time upon being oxidized by tyrosinase. *F* is the fractional activity of the partially active enzyme species. The conditions are identical as described in Figure 1.

tion of the dehydrogenase enzymes at 340 mμ. A Cary 15 spectrophotometer was used to measure the aldolase reaction. The reaction of the hydrolysis product of fructose 1,6-diphosphate and hydrazine was measured at 240 mμ and 30° (Dreshsler *et al.*, 1959). Alcohol dehydrogenase activity was measured by the method of Racker (1950). Lactic acid dehydrogenase activity was measured by the assay procedure of Schwert and Hakala (1952). Glutamic acid dehydrogenase activity was measured by Frieden (1959).

The experiments were carried out in the following manner. The substrate (the protein under study) was incubated with tyrosinase in phosphate buffer, pH 7.5, at 8°. The usual reaction mixture contained sufficient protein to be 10^{-4} M in tyrosine, tyrosinase, and buffer. A control, containing only the protein substrate, was run simultaneously. At various times throughout the reaction period, aliquots of both the control and tyrosinase-treated sample were removed and suitable dilutions were made for absorbance measurements at 280 mμ and enzyme activity assays. Oxygen consumption was measured in a Warburg apparatus at 30° using air as the gas phase.

A modified method of Folin–Ciocalteu (1927) for tyrosine determination was used. A sample (0.5 ml) was mixed with 2.0 ml of 1 N NaOH and 0.1 ml of Folin–Ciocalteu's reagent. Tyrosine (3.3 μg) gave a reading of 0.070 absorbance unit at 660 mμ.

Results

Oxidation of Yeast Alcohol Dehydrogenase by Tyrosinase. Yeast alcohol dehydrogenase is extensively oxidized by tyrosinase and this oxidation of the tyrosine residues results in the partial loss of enzymic activity. Oxygen consumption data as measured by the Warburg apparatus indicate that a limiting value of 42 moles of oxygen are consumed/mole of alcohol dehydrogenase. Therefore, 83% of the 50 tyrosine residues present in native alcohol dehydrogenase are oxidized by tyrosinase.

Figures 1 and 2 show, respectively, the rates of oxidation of the tyrosine groups in alcohol dehydrogenase as measured by the change in optical density at 280 mμ and the rate of loss of enzymic activity accompanying this oxidation. From Figure 1, it is seen that the tyrosine residues are resolved into two groups with rate constants of 2.3×10^{-2} and 1.0×10^{-3} min^{-1}, respectively. From the intercepts of Figure 1, it is estimated that 30% of the reacting tyrosine groups are oxidized at the faster rate, while the remaining 70% react at the slower rate. The data in Figure 2 show that alcohol dehydrogenase loses activity upon oxidation by tyrosinase. From the semilog plot of $Act./Act._0$ *vs. t*, it is seen that a partially active species is formed

which is only 49% as active. A semilog plot of (Act./Act.$_0$ − F)/(1 − F) *vs.* t, where F is the fractional activity of the modified species, as proposed by Ray and Koshland (1961) yields a straight line with a calculated rate constant for loss of enzyme activity of 2.0 × 10^{-3} min^{-1} at a temperature of 8°. Figure 3 is a semilog plot of the rate of oxidation of alcohol dehydrogenase as measured by oxygen consumption at 30°, and it is seen that the reactivities of the tyrosine residues can be resolved into reactions of two different rate constants. Estimated from the intercepts, 24% of the tyrosine residues react with a rate constant 3.6 × 10^{-2} min^{-1} while 76% of the tyrosine groups react with a rate constant of 3.0 × 10^{-3} min^{-1}. Although the rates as measured by oxygen consumption and the change in optical density were carried out at two different temperatures, the relative rate constants are comparable when corrected for the temperature difference and the percentage of tyrosine residues reacting in each group is in suitable agreement. The kinetic parameters, V_m and K_m, of the modified alcohol dehydrogenase species were determined from v *vs.* v/s plots and compared with the control sample in Table I. Only the V_m appears to be changed after tyrosinase treatment.

TABLE I: Comparative Parameters of Tyrosinase-Oxidized Alcohol Dehydrogenase.

	ADH Control	Tyrosinase-Oxidized ADH	Tyrosinase-Oxidized ADH in Presence of NAD
K_m (NAD) (M)	4 × 10^{-4}	4 × 10^{-4}	—
V_m (NAD) (μmole min^{-1})	1.3	0.55	—
V'_m:V_m (NAD)[a]	—	0.42	—
K_m (EtOH) (M)	2.4 × 10^{-2}	3 × 10^{-2}	3 × 10^{-2}
V_m (EtOH) (μmole min^{-1})	0.47	0.20	0.22
V'_m:V_m (EtOH)[a]	—	0.43	0.48

[a] V'_m:V_m is the ratio of the modified species V'_m to the control V_m, with respect to either NAD or ethanol.

Inactivation of Rabbit Muscle Aldolase by Tyrosinase. The incubation of aldolase with tyrosinase results in considerable inactivation of aldolase. The residual activity remaining after the reaction amounts only to 20% of the initial and control values. A semilog plot of the data, according to Ray and Koshland (1961) for the inactivation process is shown in Figure 4.

There is only a slight increase in the absorption spectrum of aldolase owing to tyrosinase oxidation. From the increase in absorbance at 280 mμ, it is estimated that only two or three of the tyrosyl groups in aldolase are oxidized in this time. The extinction coefficient for aldolase ($E^{1\%}_{280}$ 9.1) and its relatively large molecular weight (149,000) make it technically difficult to estimate the number of tyrosyl groups oxidized more closely.

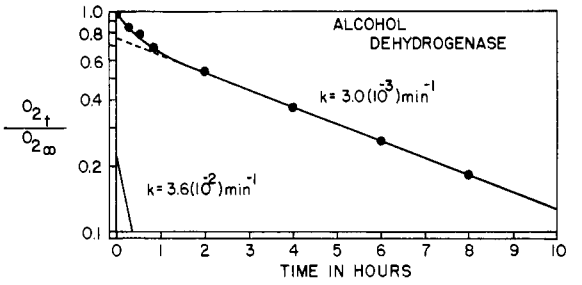

FIGURE 3: First-order plot for the oxidation of yeast ADH by tyrosinase followed by the amount of oxygen consumed. The reaction mixture contained 25 mg of ADH, 0.066 M phosphate buffer, pH 7.5, and 27 units of tyrosinase in a final volume of 3.0 ml. Oxygen consumption was measured on a Warburg apparatus at 30°.

Since it has been shown that the removal of the C-terminal tyrosine groups of aldolase by carboxypeptidase results in an enzyme species which retains only about 7% of its original activity (Dreshsler *et al.*, 1959), great care must be taken to exclude the presence of proteolytic activity in the tyrosinase preparation. Table II shows the results of an attempt to show protease activity in the tyrosinase preparation. In this experiment, a tenfold excess of tyrosinase was used to increase the ease with which the protease activity could be detected. As seen in Table I, there is

TABLE II: Determination of Protease Activity in Tyrosinase Preparation.[a]

Sample	A_{660}
Aldolase + tyrosinase	0.050
Tyrosinase-treated aldolase	0.052
Tyrosine (3.3 μg)	0.070

[a] Aldolase (2.14 mg) was incubated with tyrosinase (36 units) in sodium phosphate buffer, pH 7.4, 0.04 M at 5° in a final volume of 0.4 ml. After 1 hr, 0.4 ml of 10% trichloracetic acid was added. A control was run in which the trichloracetic acid was added to the aldolase before the addition of tyrosinase. The suspensions were filtered through a Millipore filter and the supernatants were taken for the determination of tyrosine by a modified Folin–Ciocalteu (1927) method as described in the Methods section. The absorbance at 660 mμ was measured on the expanded scale of the Cary 15. Samples were run in duplicate.

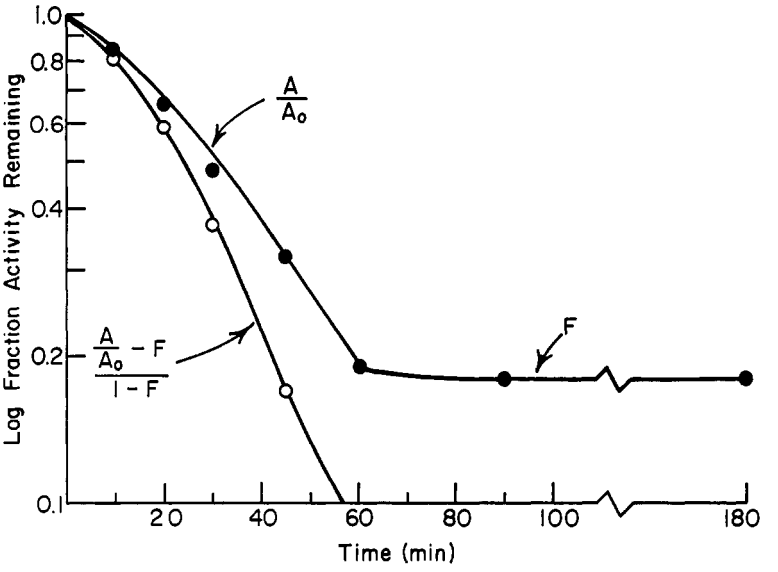

FIGURE 4: Aldolase (1.07 mg) was incubated at 5° with tyrosinase (3.6 units, 180 units/mg) in phosphate buffer, pH 7.4, 0.04 M, in a final volume of 0.20 ml. Aliquots (25 μl) were removed at various times and the aldolase activity was assayed as described in the Methods section. Controls containing no tyrosinase were run simultaneously. The data are plotted on a semilog scale according to the method of Ray and Koshland (1961).

no difference between the tyrosinase-treated aldolase and the aldolase plus tyrosinase sample. The amount of C-terminal tyrosine from the three chains of aldolase (2.14 mg) would be 7.82 μg. Since the oxidized tyrosine group gives only 60% of the color with Folin–Ciocalteu reagent as does free tyrosine, this would be equivalent to 4.7 μg of tyrosine. Under the conditions used for tyrosine determination reported in Table II, 3.3 μg of tyrosine give a A_{660} of 0.070.

If the loss of the enzymic activity of aldolase is due to protease activity, then the inactivation of tyrosinase by CN^- should not prevent inactivation of the aldolase. These results are shown in Table III. Tyrosinase-treated aldolase had only 20% activity remaining, while CN^--inactivated tyrosinase had no effect on aldolase. Cyanide had no effect on aldolase activity.

TABLE III: Effect of Cyanide on Inactivation of Aldolase by Tyrosinase.[a]

Sample	% Remaining Act.
Aldolase	100
Aldolase + CN^-	100
Tyrosinase-treated aldolase	18
CN^--inactivated tyrosinase + aldolase	94

[a] Tyrosinase (23 units, 0.42 mg) was incubated in CN^- (0.14 M) for 2 hr at 5°, at pH 7.4. A similar sample of tyrosinase, without CN^-, was kept at 5° for the 2 hr. After 2 hr, 1.07 mg of aldolase was added to the samples. A control containing no tyrosinase and a control containing aldolase and cyanide were incubated simultaneously. Aliquots (25 μl) were taken for aldolase assay after incubation of 1 hr.

Oxidation of Other Proteins by Tyrosinase. GDH is not oxidized by tyrosinase, and the enzymic activity of GDH is not affected. Even after 36 hr, the GDH which was incubated with tyrosinase had as much activity as the GDH control.

LDH is oxidized by tyrosinase, but this oxidation is slow compared to alcohol dehydrogenase. All accessible tyrosine residues are oxidized at apparently the same rate. The pseudo-first-order rate constant, determined from a semilog plot of $(A_\infty - At)/(A_\infty - A_0)$ *vs.* t, is 4.2×10^{-4} min^{-1}. It is estimated from the change in extinction coefficient at 280 mμ that only four tyrosine residues of the 40 present in LDH are oxidized by tyrosinase. The rate of oxidation of LDH is not affected by the presence of 1×10^{-4} M NAD. The enzymic activity of LDH is not affected by this limited oxidation by tyrosinase.

Under the conditions used, none of the 25 tyrosine residues of glyceraldehyde 3-phosphate dehydrogenase are oxidized by tyrosinase. Soybean trypsin inhibitor was tested as a substrate for tyrosinase. Soybean trypsin inhibitor is oxidized by tyrosinase, giving the characteristic change in absorption. From the change in extinction coefficient at 280 mμ, it is estimated that two of the four tyrosine residues in soybean trypsin inhibitor are susceptible to tyrosinase oxidation. The biological activity of oxidized soybean trypsin inhibitor, that is its ability to inhibit trypsin activity, was tested (Schwert, 1955), and found to be no different from native soybean trypsin inhibitor. Met-myoglobin, a protein of molec-

ular weight 18,000 and containing three tyrosine residues, is not oxidized by tyrosinase under the conditions used in these experiments.

Discussion

The use of tyrosinase as a specific amino acid reagent for tyrosyl residues in proteins may give considerable information concerning the role of the tyrosine side chain in the structure and biological activity of proteins. Three distinct groups of proteins are encountered experimentally with respect to reactivity to tyrosinase: (1) proteins, in which the biological activity is lost, either partially or completely, on oxidation to tyrosinase; (2) proteins, in which tyrosyl groups are oxidized, but there is no loss of biological activity; and (3) proteins which are completely resistant to oxidation by tyrosinase. The proteins which fall into each of these categories are summarized in Table IV.

TABLE IV: Proteins Tested as Substrates for Tyrosinase.[a]

Proteins Oxidized by Tyrosinase
(1) Biological activity reduced: alcohol dehydrogenase, aldolase oxytocin, vasopressin, rennin, invertase, fibrinogen, and thrombin
(2) Biological activity effect absent or unknown: insulin,[b] lactic dehydrogenase, soybean trypsin inhibitor, and bovine serum albumin
Proteins Not Susceptible to Tyrosinase
(3) Ribonuclease, glutamic dehydrogenase, glyceraldehyde 3-phosphate dehydrogenase, lysozyme, pepsin, trypsin, chymotrypsin, catalase, and lactoglobulin

[a] See text for appropriate references. [b] Biological activity of oxidized insulin has not been determined.

In the work presented here, it is shown that ADH is extensively oxidized by tyrosinase. Approximately 42 tyrosyl groups of ADH are oxidized resulting in loss of ADH activity (50%). The rate constants for the oxidation of the tyrosyl groups by tyrosinase were 2.3×10^{-2} and 1×10^{-3} min^{-1}. Treatment of the data according to Ray and Koshland (1961) gives a rate constant for the loss of alcohol dehydrogenase activity of 2.0×10^{-3} min^{-1}. If the tyrosine groups are essential for biological activity, then the rate constant for activity loss cannot be less than the rate constant for tyrosine oxidation. It is, therefore, concluded that the "slow" tyrosine groups are involved in the enzymic activity of ADH.

Table I is a summary of the kinetic parameters of V_m and K_m obtained from native and tyrosinase-treated alcohol dehydrogenase. There is no significant change in K_m with respect to either NAD or ethanol. Only the V_m changes and the ratio $V'_m:V_m$ is in good agreement with the limiting value of F, the fractional activity, found from a semilog plot of Act./Act.$_0$ *vs.* t (Figure 2). Since the loss of ADH enzyme activity cannot be prevented by the addition of NAD to the reaction mixture, it is implied that the tyrosine groups are not involved in the binding of NAD. The tyrosine residues are probably involved in the conformation of the active site of the alcohol dehydrogenase molecule through hydrogen bonding of the phenolic group. An alternative explanation is that the oxidized species might affect the V_m by inhibiting the activity of the remaining unoxidized enzyme perhaps by competing for substrate as does the "enzymoid" of lysozyme (Frieden, 1956). We regard this as unlikely in view of the fact that over 80% of the tyrosines of yeast alcohol dehydrogenase are oxidized with no change in K_m.

Another example of this group is rabbit muscle aldolase. There is a very limited oxidation of the tyrosyl groups of aldolase by tyrosinase. Of the 42 tyrosine groups in aldolase, it is estimated that only a maximum of 4 are oxidized. However, incubation of aldolase with tyrosinase results in the loss of 82% of aldolase activity. Dreshsler *et al.* (1959) have shown that carboxypeptidase treatment of aldolase results in the release of three C-terminal groups. Removal of the C-terminal tyrosyl residues resulted in the loss of 93% of aldolase activity. Therefore, extreme care was taken to rule out the presence of a protease in the tyrosinase preparations used. The results of experiments designed to rule out the presence of protease activity are presented in Tables II and III. These results indicated that the loss of aldolase activity is not the result of the rupture of the tyrosyl peptide bond, but due to the alteration of the phenolic side chain. Wu and Shi (1964) have concluded that the C-terminal tyrosyl residues of aldolase are involved in the binding of the 6-phosphate groups of the fructose 1-diphosphate molecule. Presumably the oxidation of the C-terminal tyrosyl groups of aldolase by tyrosinase results in the formation of C-terminal dopa-quinone residues of aldolase, which would not be expected to bind in the same manner as the phenolic group.

Other examples of this group of proteins have been reported. Sizer and Wagley (1951) showed that fibrinogen was oxidized by tyrosinase and the oxidized fibrinogen could not be converted to fibrin. Sizer *et al.* (1949) also reported on the agglutination of red blood cells by tyrosinase. Recent studies have shown blood cells are not only agglutinated but are, in addition, lysed (Cory, 1967).

In the second group of proteins are lactic acid dehydrogenase and soybean trypsin inhibitor. Oxidation of four tyrosyl groups in LDH has no effect on the enzymic activity of LDH. Two of the four tyrosyl residues in soybean trypsin inhibitor are oxidized by tyrosinase, but the inhibitory activity of trypsin inhibitor to trypsin activity is not affected. These results suggest that the tyrosyl groups are accessible to the protein surface, but not necessary for biological activity. Insulin

is included in this group because the biological activity of the oxidized insulin species has not been determined. Other proteins, with no specific biological activity, but which are substrates for tyrosinase, should be included in this group. These include albumin (Frieden *et al.*, 1959) and α-lactalbumin (Yasunobu *et al.*, 1959).

The third group of proteins are the proteins which in the native state are not substrates for tyrosinase. From the work presented here, glyceraldehyde 3-phosphate and glutamic acid dehydrogenases are not substrates for tyrosinase. In addition, it has been shown that ribonuclease (Frieden *et al.*, 1959) and lysozyme (Frieden *et al.*, 1959) are not susceptible to tyrosinase oxidation. However, when ribonuclease is treated with performic acid, oxidizing the disulfide bonds, the modified ribonuclease becomes a very good substrate for tyrosinase and all six of the tyrosine residues are oxidized (Frieden *et al.*, 1959; Lissitzky, 1962). Lysozyme, on the other hand, when treated with either performic acid (Frieden *et al.*, 1959) or urea (Yasunobu and Wilcox, 1948) still does not become susceptible to tyrosinase. It is concluded by these authors that the inaccessibility of the tyrosine residues of native ribonuclease arises out of the tertiary structure while the inaccessibility of the tyrosine residues of lysozyme is due to its primary structure. Results obtained with tyrosinase on native ribonuclease (Frieden *et al.*, 1959) do not correlate with the results on studies of "exposed" tyrosyl groups in ribonuclease by titration (Cha and Scheraga, 1963) or difference spectroscopy (Herskovits and Laskowski, 1962). In addition, the results on insulin oxidation by tyrosinase (Cory and Frieden, 1967) do not correlate with the results obtained on insulin iodination (Springell, 1962), solvent perturbation (Herskovits, 1965), and acetylation (Riordan *et al.*, 1965). Cha and Scheraga (1963) have concluded that the term "buried" tyrosine means something different for each method employed.

Even though enzymic modification of amino acid side chains would be the most specific and gentle method of side-chain alteration, the use of tyrosinase as a specific reagent for tyrosyl groups has certain limitations. An active site could have a tyrosyl residue which is intimately involved in the biological activity of the protein, but not accessible to tyrosinase. In this case, negative results are not useful. On the other hand, because of the mild conditions and the gentleness and specificity of the method, positive results should have some significance with reference to protein structure.

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