Oxidation of Insulin by Tyrosinase*

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The oxidation of the tyrosyl groups of insulin by mushroom tyrosinase has been reinvestigated. Typical spectral changes have been observed in the ultraviolet region. Oxygen consumption data indicate that probably all four of the tyrosyl residues of insulin are oxidized. Relative initial rates of oxidation were as follows: B chain > amorphous insulin > A chain > Zn-insulin. The tyrosinase preparation used was devoid of detectable protease activity, and no proleolytic degradation of the oxidized insulin was observed.

It has been stated by Haas *et al.* (1951) that "crystallized insulin is relatively very resistant to tyrosinase and the small changes in absorption spectrum are scarcely more than could be accounted for by the autooxidation of the tyrosine alone," and by Yasunobu *et al.* (1959), that "insulin, which has a molecular weight of 6,000, was relatively inert to tyrosinase-catalyzed oxidation, in agreement with the results of Haas, *et al.*" In his review on the oxidation of proteins by tyrosinase, Sizer (1953) states that "native insulin, while not completely refractory, is rather resistant to attack by tyrosinase. When insulin is partially hydrolyzed by pepsin or chymotrypsin and the resulting small fragments removed by dialysis, the insulin is very readily and extensively oxidized by tyrosinase."

However, when a different preparation of tyrosinase became available (Frieden and Ottesen, 1959), we obtained evidence for the susceptibility of the tyrosine groups of insulin to oxidation by mushroom tyrosinase. In this paper, we present data on the oxygen uptake and spectral changes observed when insulin is exposed to higher activity levels of a purified, protease-free preparation of mushroom tyrosinase.

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EXPERIMENTAL

Materials.—Purified mushroom tyrosinase was prepared in our laboratory by essentially the procedure of Frieden and Ottesen (1959). The enzyme preparation had a specific activity of 320 catecholase units/E'_{280} and 80 *p*-cresolase units/E'_{280}.[1] The sample showed only one peak in the ultracentrifuge. The insulin preparations were generously supplied by Eli Lilly and Company through the courtesy of Drs. O. Behrens and H. Higgins. Zn-insulin, lot 693502; amorphous insulin, lot W1282; A-chain, lot 190-400B-28A; and B-chain, lot 190-400B-28B, were used in this study. None of the results reported below was affected by dialysis of the insulin before use.

Methods.—Spectral changes were observed on a Beckman DK Spectrophotometer. The reaction mixtures contained substrates (0.40 μ-moles in tyrosine), 1.00 ml of 0.05 M phosphate buffer, *p*H 7.4, enzyme (225 catecholase and 60 *p*-cresolase units), and H_2O to total volume of 3.00 ml. The reference cell contained all components except substrate. The reaction mixtures were kept at 30°. Absolute optical density readings were made on a Beckman DU Spectrophotometer.

The rate of oxidation of insulin at 30° in the presence of tyrosinase was studied by following the increase in optical density at 280 mμ. The reaction mixture was the same as that used in the spectral change study. The spectrophotometer was zeroed against the substrate, and the blank

[1] E'_{280} is the corrected O.D. reading according to the empirical formula of Eiger and Dawson (1949). If the value of 14.1 is assumed to be correct for the $E_{1\%}$ value then the activity of a tyrosinase preparation used is 420 catecholase units/mg and 113 *p*-cresolase units/mg. However, Kertesz and Zito (1957) have reported an $E_{1\%}$ value of 27.6 for homogeneous tyrosinase. If we use the Kertesz and Zito value, this would about double the activities to 822 catecholase units/mg and 221 *p*-cresolase units/mg. The purified tyrosinase used here probably resembles the Kertesz and Zito preparation more closely, since extraneous chromogenic material is eliminated by chromatography on DEAE-cellulose.

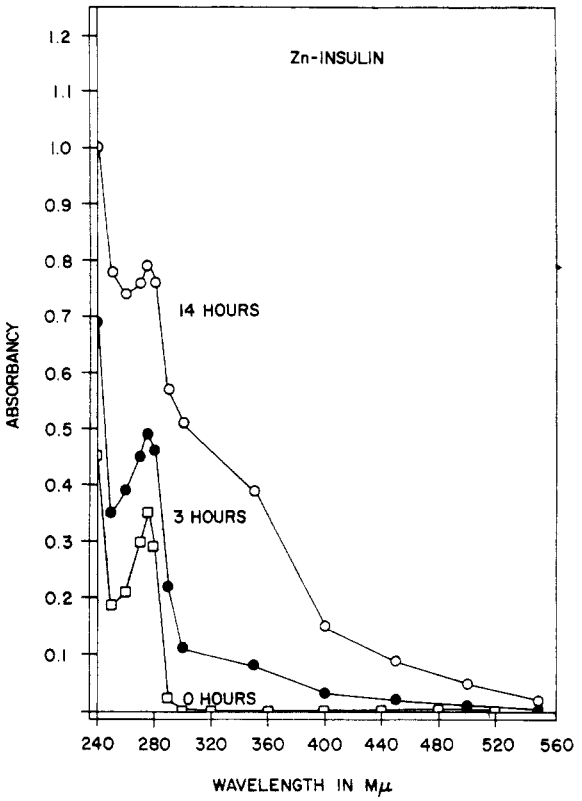

FIG. 1.—The spectral changes observed for Zn-insulin on oxidation by mushroom tyrosinase. Conditions as described in Methods section.

containing enzyme was subtracted from the subsequent readings. The optical density change of the enzyme over the reaction period when no substrate was present was 0.002.

Oxygen uptake was measured at 30° in the Warburg apparatus with air as the gas phase in 1.0-ml or 3.0-ml vessels. The substrate concentration was 4.0 μmoles in tyrosine; 200 catecholase units of tyrosinase were used.

Protease activity was determined by the methods of Anson (1938) and Kunitz (1947), except that insulin was used as substrate and the sample was tested after the maximum incubation time of 30 hours.

Electrophoresis of products was carried out in a Spinco Model R apparatus. The samples were concentrated by isoelectric precipitation of the insulins. The precipitates were then taken up in 0.2 ml of phosphate buffer, *p*H 7.6. Thirty μl of this was then used. Controls were carried through the above procedure. The electrophoresis was carried out for 12 hours in *p*H 8.6 barbital buffer, 0.75 M, at 3.0 mamp.

For ultracentrifugal studies, a 1% solution of insulin in 0.010 M phosphate buffer, *p*H 7.4, and 0.14 M NaCl was centrifuged at 59,780 rpm in a Spinco Model E instrument.

RESULTS

Oxidation of Zn-Insulin and Amorphous Insulin.—Figures 1 and 2 show the spectral changes observed for Zn-insulin and amorphous insulin. The changes which are observed in the spectra are more than could be accounted for by autooxidation of the purified enzyme used. The changes in spectra are consistent with those which would be expected for a susceptible protein or polypeptide as reported by Yasunobu *et al.* (1959) and observed in this laboratory.[2]

Relative Rates of Oxidation of Insulins and Insulin Chains.—Figure 3 shows the relative rates of oxidation of insulin samples as determined by the change in optical density at 280 mμ. Fredericq (1956) has shown that the presence of Zn in insulin increases the size of the molecular species, as indicated by an increased sedimentation constant. The larger molecular species probably makes the tyrosyl residues of Zn-insulin less accessible than those of amorphous insulin, thereby accounting for the difference in oxidation rates.

In view of the greater rate of oxidation of the B chain of insulin compared to the A chain shown in Figure 3, it is conceivable that at least one of the tyrosine groups of the A chain is sterically or otherwise hindered. Springell (1961) has suggested that at least one tyrosine of the B chain is masked from iodination at an acid *p*H. This masking does not appear to be significant in the action of tyrosine on the B chain at *p*H 7.4.

The lower apparent maximum absorbance observed in the oxidation of the A chain after eight hours of contact with tyrosinase may be due to the presence of non-peptide material in the A chain preparation. In these experiments, weights of insulin or its constituent chains corresponding to equal moles of tyrosine were used.

Oxygen Consumption.—Table I reports the moles of oxygen consumed per mole of insulin at various times throughout the reaction period.

[2] Karin Forsblad and Earl Frieden, unpublished experiments. Early in 1959 we independently obtained data similar to those reported by Yasunobu *et al.* (1959) in which the oxidation of a variety of amino-terminal, carboxyl-terminal, and internal tyrosine peptides in the presence of tyrosinase were compared. We confirm their observation that tyrosinase may be used to detect the presence of small quantities of amino-terminal tyrosine in peptides. The spectra of oxidized amino-terminal tyrosines were substantially different from those of oxidized carboxyl-terminal and internal tyrosines. The increases in extinction at 300 mμ for the L-tyrosine amide, L-tyrosine ethyl ester, and L-tyrosine hydrazide were equivalent to L-tyrosine oxidation. Carboxyl-terminal tyrosine peptides gave about one-half this change after tyrosinase treatment. This included peptides such as glycyl-tyrosine, leucyl-tyrosine, *N*-acetyl-L-tyrosine, carbobenzoxyglutamyl-tyrosine, glycyl-tyrosine amide acetate, benzoyl-L-tyrosine amide, *N*-acetyl-L-tyrosine ethyl ester, and the proteins: insulin, A and B chains of insulin, bovine plasma albumin, pepsin, and others.

These results show clearly that all four of the tyrosyl residues of insulin are oxidized. This calculation assumes that one mole of oxygen is consumed per mole of tyrosine in the protein (Yasunobu and Dandliker, 1957; Lissitzky *et al.*, 1960).

TABLE I

OXYGEN UPTAKE IN THE TYROSINASE-CATALYZED OXIDATION OF INSULIN

Moles of Oxygen Consumed per Mole of Insulin

Time (hr.)	Zn-Insulin		Amorphous Insulin	
	Experiment[a]			
	1	2	1	2
2	—	0.1	—	0.2
5	0.8	—	1.2	—
8	—	0.7	—	1.7
12	1.6	—	3.2[b]	—
17	—	2.0	—	2.6
25	2.8[b]	—	—	—
29	—	3.1	—	3.2
36	—	3.4	—	3.8
40	—	3.5	—	3.8

[a] Experiment 1 in duplicate, experiment 2 in triplicate. [b] No further measurements taken, though reaction was not complete at these times.

Electrophoresis of Insulin and Oxidized Insulin.—The oxidized products moved faster than their control counterparts toward the positive pole. In both cases the oxidized insulins moved about 35% faster than the native insulins.

Determination of Protease Activity in Tyrosinase Preparation.—The reaction mixture was used to determine if any proteolysis occurred with our enzyme preparation. At 280 mμ the trichloroacetic acid supernatant was read directly; at 750 mμ Folin-Ciocalteu's reagent was added prior to determination of the optical densities. Table II shows these results. The differences observed are negligible, showing that the tyrosinase preparation was devoid of detectable protease activity. The reaction samples after the reaction period did not decrease in optical density after dialysis.

TABLE II

DETERMINATION OF PROTEASE ACTIVITY BY THE METHOD OF ANSON AND KUNITZ (1938)

The trichloroacetic acid supernatant was read at 280 mμ. The sample was then prepared for the Folin-Ciocalteu tyrosine test and read at 750 mμ. The control contained all the components except enzyme.

	280 mμ	750 mμ
Control	0.070 ± 0.005[a]	0.035 ± 0.001
Control + tyrosinase	0.100 ± 0.010	0.038 ± 0.002

[a] Standard deviation of the optical density measurements.

Further evidence for the absence of demonstrable protease activity in these tyrosinase preparations may be deduced from the fact that several proteins were not oxidized. Tyrosinase had no effect on lysozyme or lactoglobulin and gave even less oxidation of ribonuclease after prolonged incubation with tyrosinase; these facts were also noted by Yasunobu and Dandliker

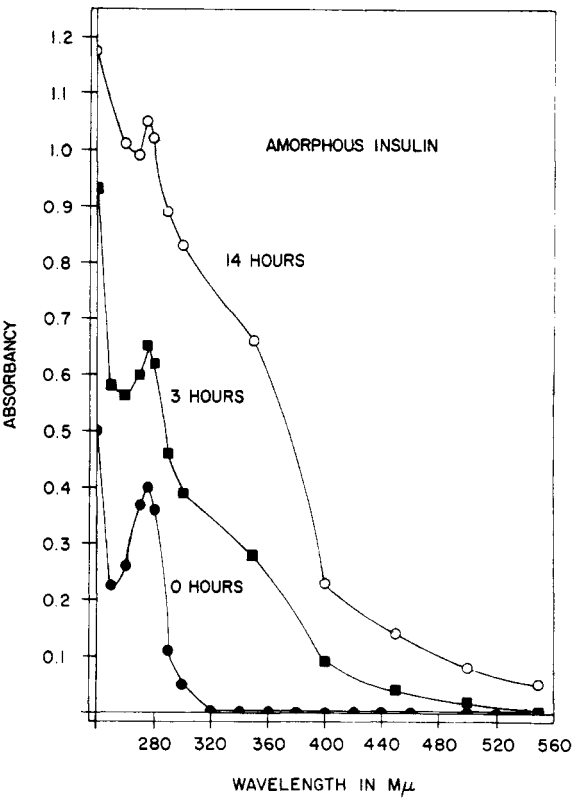

FIG. 2.—The spectral changes observed for amorphous insulin on oxidation by mushroom tyrosinase. Conditions as described in Methods section.

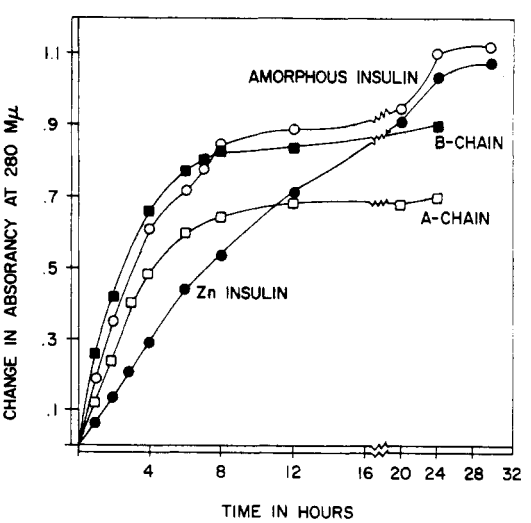

FIG. 3.—The relative rates of oxidation of insulin and its constituent chains by mushroom tyrosinase as measured by the change in optical density at 280 mμ. Conditions as described in Methods section.

(1957). It is reasoned that if cleavage of any of these three proteins had occurred, some oxidation by tyrosinase might have been observed.

Ultracentrifugal Analysis.—Ultracentrifugal analysis showed the products to be homogeneous. The sedimentation constants of the products did not differ from that of the native insulin. The absence of protease activity is indicated in the experiments described above.

Discussion

Possible Explanation for Different Observations on the Susceptibility of Insulin to Mushroom Tyrosinase.—Neither Haas *et al.* (1951) nor Yasunobu *et al.* (1959) could obtain evidence for the catalysis of the oxidation of the tyrosyl groups in insulin by mushroom tyrosinase. We believe that our data clearly establish that tyrosinase does act on insulin. These differences may be accounted for in terms of differences in the tyrosinase preparations or in the properties of insulin or perhaps even in the conditions of the test.

It is quite conceivable that the susceptibility of insulin to tyrosinase action might vary with different tyrosinases resulting from different methods of preparation. The tyrosinase used in these experiments was prepared by a rather different method than the enzyme used by Haas *et al.* (1951) and Yasunobu *et al.* (1959). Our enzyme preparations had a relatively high *p*-cresolase activity. It is therefore possible that this high monophenolase activity might catalyze a more rapid oxidation of the tyrosyl residues of insulin. The possibility of the existence of separate "cresolase" and "catecholase" components of the "phenolase complex" of mushrooms has been renewed by Dressler and Dawson (1960). The multiplicity of mushroom tyrosinase is also suggested by the recent work of Smith and Kreuger (1961). Earlier Mason (1956) had commented on the possible changes in the configuration and substrate specificity of tyrosinase during isolation.

In a comparative study of the rates of oxidation of several different proteins, including bovine plasma albumin, pepsin, growth hormone, and other proteins (Frieden *et al.*, 1959), insulin was found to give the slowest rate of oxygen uptake and the slowest change in the ultraviolet spectrum. Thus, in the present investigation, by using tyrosinase with a higher level of "cresolase" activity, we were able to better detect the response of insulin.

Differences in the susceptibility of insulin to different preparations of tyrosinase could be explained further if we assume that the earlier tyrosinase preparations had more or different extraneous proteins which were susceptible to oxidation. Since reaction inactivation of this enzyme is well known (Miller and Dawson, 1941), the products of the oxidation of these readily oxidizable protein impurities might have inactivated the tyrosinase. Thus the more slowly oxidized insulin would be exposed to less and less active tyrosinase as the impurities produced more product inhibition.

An important difference in the experiments may have been the *p*H at which the oxidations were conducted. It is desirable to stay in the optimum range of tyrosinase, *p*H 6–8, but it is equally necessary to maintain the *p*H at 7.0 or above, since insulin is practically insoluble in the *p*H range 4.5 to 7.0 (Fredericq and Neurath, 1950). Haas *et al.* (1951) buffered their solutions at *p*H 7.5 with ammonium acetate, which is out of the range where this buffer would be effective. Yasunobu *et al.* (1959) buffered their solutions at *p*H 6.8, slightly outside the preferred solubility region of insulin. In our experiments, phosphate buffer at *p*H 7.4 was used. No *p*H change during the experiment or insolubilization of insulin was observed.

It is also conceivable that there might be conformational differences in the insulin preparations. We have observed tyrosinase action on at least four different insulin preparations, although not all these data are included here.

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