Faster Oxidation of Tyrosine-26 of Oxidized B Chain of Insulin by Tyrosinase*

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ABSTRACT: The oxidation of the tyrosyl residues of bovine insulin is catalyzed by tyrosinase [Cory, J. G., Bigelow, C. C., and Frieden, E. (1962), *Biochemistry 1*, 419]. All four of the tyrosyl groups are oxidized at the same rate in zinc–insulin, while in amorphous insulin one of the tyrosyl groups reacts four times faster than the other three tyrosyl groups. The tyrosyl residues of performic acid oxidized A chain react at the same rate, but one of the tyrosyl groups of performic acid oxidized B chain reacts 13 times faster than the other tyrosyl group. Partial oxidation of oxidized B chain with tyrosinase, followed by trypsin hydrolysis, separation of the resulting heptapeptide, and amino acid analysis, has permitted the identification of tyrosine-26 of oxidized B chain as the more rapidly oxidizable tyrosine.

The "states" of tyrosyl residues in proteins have been the subject of many studies. These studies have included titration, iodination, and the solvent perturbation technique. Cha and Scheraga (1963a) studied the titration and iodination of ribonuclease and concluded that the number of abnormal ionizing tyrosyl groups were not the same as the number of uniodinated tyrosyl groups. The study was extended to pinpoint the location fo the unreactive tyrosyl residues in ribonuclease (Cha and Scheraga, 1963b). Herskovits and Laskowski (1962a,b) have studied the effect of various solvents on the difference spectra of several proteins to ascertain the location of chromophoric side chains (tyrosine and tryptophan). Cyanuric fluoride (Kurihara *et al.*, 1963) and *N*-acetylimidazole (Riordan *et al.*, 1965) have been used recently as specific reagents for tyrosyl residues in proteins.

Tyrosinase has been used by several groups (Sizer, 1953; Yasunobu *et al.*, 1959; Lissitzky *et al.*, 1960; Frieden *et al.*, 1959) to investigate the accessibility of the tyrosyl groups to the surface of the protein and to determine if these tyrosyl groups are in the active site. It has been previously reported that the tyrosyl groups of insulin are oxidized by tyrosinase (Cory *et al.*, 1962). In this communication, we report on the differential reactivities of the tyrosyl groups of insulin and its chains to tyrosinase. The location of the more rapidly oxidized tyrosyl groups has been determined as tyrosine-26 in the case of the oxidized B chain.

Experimental Section

Materials. The tyrosinase (*o*-diphenol:oxygen oxidoreductase, EC 1.10.3.1) used in these experiments was prepared from fresh mushrooms essentially by the procedures of Frieden and Ottesen (1959). The changes in the procedure were: (1) precipitating the tyrosinase from the crude 30% acetone extract with absolute ethanol instead of acetone; (2) immediately making the solution up to 1% in calcium acetate instead of doing it in two steps; (3) precipitating the calcium acetate supernatant with ammonium sulfate and carrying out dialysis after the ammonium sulfate precipitate is taken up in phosphate buffer; (4) not dialyzing the concentrated tyrosinase preparation against sodium chloride.

The tyrosinase prepared by this procedure was ultracentrifugally homogeneous with a sedimentation constant at 20° of 4.1 S at 0.5% concentration. The enzyme sample was electrophoretically homogeneous in 0.075 M barbital buffer, pH 8.6. This preparation apparently differs from the preparations of Bouchilloux *et al.* (1963). Kertesz and Zito (1962), and Mallette and Dawson (1949) in that their reported s_{20} is about 6.4 and also differs from the preparation of Smith and Krueger (1963). However, earlier Kertesz and Zito reported an active species with an s_{20} of 2.7 (Kertesz and Zito, 1957). The lower sedimentation constant obviously also differs from a previously reported value of 7.3 S prepared by a similar but not identical method from mushrooms from a different source. Our preparation was devoid of detectable protease activity, tested as before (Cory *et al.*, 1962).

The 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 phos-

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[1] Abbreviations used: L-dopa, 3,4-dihydroxy-L-phenylalanine; dopachrome, 2-carboxy-2,3-dihydroindole-5,6-quinone.

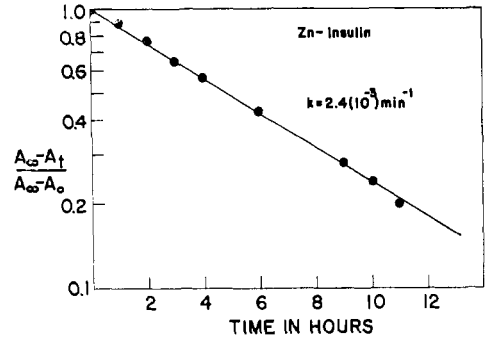

FIGURE 1: First-order plot for the oxidation of zinc–insulin by mushroom tyrosinase. A_∞, maximum absorbance at 280 mμ determined at infinite time. A_0 and A_t, absorbance at 280 mμ at time 0 and t, respectively. Thus, the ratio $(A_\infty - A_t):(A_\infty - A_0)$, represents the fraction of unoxidized tyrosine at time t. Insulin concentration was 10^{-4} M with respect to tyrosine in phosphate buffer, pH 7.5. The reaction mixture contained 36 units of tyrosinase/ml. The change in optical density was followed at 280 mμ and 30°.

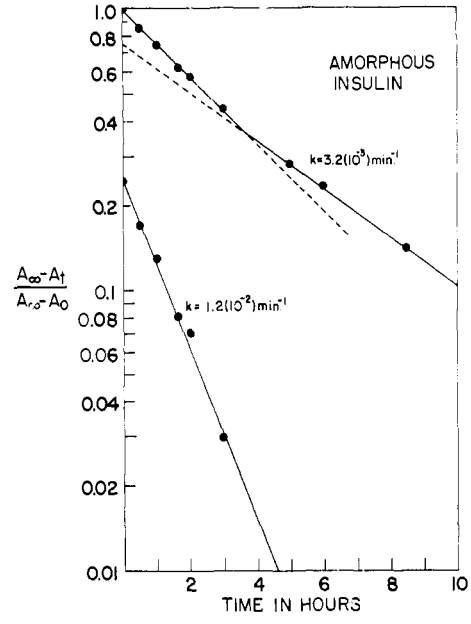

FIGURE 2: First-order plot for the oxidation of amorphous insulin by tyrosinase. Conditions are the same as for zinc–insulin (Figure 2).

phate, 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.

The insulin samples, zinc–insulin (lot 693502), amorphous insulin (lot W1282), performic acid oxidized A chain (lot 190-400B-28A), and performic acid oxidized B chain (lot 190-400B-28B), were gifts of Eli Lilly and Co. Additional oxidized B chain of insulin was purchased from Mann Research Laboratories, Inc. The complete amino acid analysis of oxidized B chain agreed closely with the values reported by Sanger and Tuppy (1951). Tyrosine values were 1.50 ± 0.07 moles/B chain (five analyses). This low value is probably due to degradation of the tyrosine by performic acid (Sanger and Tuppy, 1951).

Methods. The rates of oxidation of the tyrosine groups of the insulins were measured at 280 mμ at 30° in either a Beckman DU spectrophotometer or a Cary 15 recording spectrophotometer. The reaction mixtures contained substrate (the insulin sample) 1×10^{-4} M in tyrosine, sodium phosphate buffer, 0.066 M, pH 7.5, and tyrosinase (36 EC units/ml).

For determination of the location of the "fast" tyrosine residue in the B chain, 10 mg of Mann B chain in sodium phosphate buffer, pH 7.5, was treated with tyrosinase (65 EC units). The reaction was allowed to continue until a desired OD_{280} change was attained.[2]

The oxidation was stopped by the addition of sufficient HCl to bring the solution to pH 2 to denature the tyrosinase. After standing for 2–3 hr, the pH of the solution was readjusted to 7.5. Trypsin (100 μg) was added and hydrolysis was allowed to continue for 3–4 hr. Tryptic hydrolysis was stopped by the addition of HCl. Controls containing B chain, but not treated with tyrosinase, were simultaneously subjected to trypsin hydrolysis.

Aliquots were removed from both the control and tyrosinase-treated samples for complete acid hydrolysis and amino acid analysis. The remaining samples were lyophilized, redissolved in 0.5 ml of H_2O, and streaked on Whatman No. 3 filter paper (18 × 22 in.). The tryptic hydrolysates were separated by high-voltage paper electrophoresis according to Sanger and Tuppy (1951) in a Gilson Model D-HVE unit. The heptapeptide was located by ninhydrin, eluted, and subjected to complete acid hydrolysis simultaneously. In agreement with Sanger and Tuppy (1951), we found that trypsin hydrolysis of insulin B chain results in cleavage of the Arg-22 and Lys-29 bonds giving the heptapeptide (23–29) containing Tyr-26. Amino acid analyses were run in duplicate in all cases on a Spinco amino acid analyzer.

Results

Oxidation of Insulin by Tyrosinase. The four tyrosine residues of insulin have been previously shown to be susceptible to oxidation by tyrosinase (Cory *et al.*, 1962). However, in the present experiments we have used a higher concentration of tyrosinase. Figure 1

[2] The increase in extinction coefficient ($\Delta\epsilon_{280}$) at 280 mμ for the oxidation of a tyrosyl group (non-N terminal) is 4.0 ± 0.1 (10^3). This value was determined by measuring the change in OD_{280} and the corresponding oxygen consumed. One mole of O_2 is consumed per mole of tyrosyl residue oxidized (9).

TABLE I: Amino Acid Analyses of Control and Tyrosinase-Treated B Chain and Its Heptapeptide.[a]

Amino Acid	Incubation Time (18 min)				Incubation Time (30 min)			
	Intact B Chain		Heptapeptide[b]		Intact B Chain		Haptapeptide[b]	
	Control	Tyrosinase Treated	Control	Tyrosinase Treated	Control	Tyrosinase Treated	Control	Tyrosinase Treated
Asp	1.14	1.22			0.98	1.04		
Thr	0.84	0.89	0.83	1.17	0.90	0.89	1.20	1.18
Ser	0.83	0.95			0.91	1.20		
Glu	3.03	3.14			3.10	3.04		
Pro	1.14	0.98	1.39	1.35	1.00	0.94	1.25	1.30
Gly	3.00	3.00	1.00	1.00	3.00	3.00	1.00	1.00
Ala	1.96	2.06			1.90	1.89		
Val	2.95	3.04			4.07	2.84		
Leu	3.74	3.91			4.07	3.90		
Tyr	1.40	1.05	0.86	0.49	1.60	0.79	1.08	0.50
Phe	2.62	2.56	1.77	1.20	2.82	2.62	1.60	0.90

[a] B Chain (10 mg) was incubated at 30° with tyrosinase (65 EC units) in sodium phosphate buffer, pH 7.5, for 18 and 30 min, respectively. The reaction was stopped by addition of acid, the samples were treated with trypsin and subjected to electrophoresis as described in the text under Methods to obtain the heptapeptide. Data are given in moles of amino acid per polypeptide chain. [b] Amino acids 23–29.

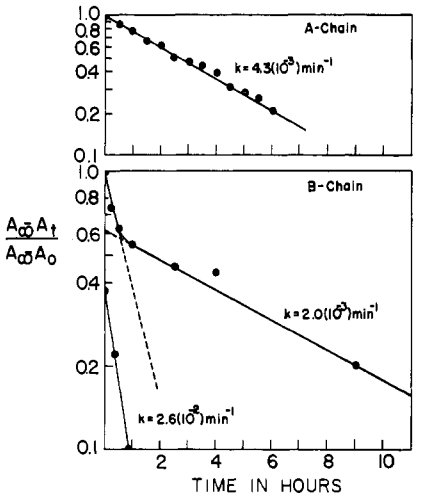

FIGURE 3: First-order plots for the oxidation of the A and B chains of insulin by tyrosinase. Conditions are the same as for zinc–insulin (Figure 2).

shows a semilog plot for the oxidation of zinc–insulin by tyrosinase as measured by following the change in optical density at 280 mμ. The curve is linear with no breaks indicating that all four of the tyrosyl residues are oxidized at the same or very nearly the same rate. The pseudo-first-order rate constant for the oxidation of tyrosyl residues in zinc insulin as determined from the slope is 2.4×10^{-3} min^{-1}. Figure 2 shows a semilog plot for amorphous insulin. However, in this case there is a definite curvature yielding two linear portions from which two rate constants are determined. The pseudo-first-order rate constants are: for the "fast" tyrosine, 1.2×10^{-2} min^{-1}; for the "slow" tyrosines, 3.2×10^{-3} min^{-1}. From the intercepts of the lines on the vertical axis, it was estimated that three of the four tyrosyl residues react at the same or nearly the same rate while the remaining tyrosyl group reacts four times faster than the other three.

Figure 3 shows the semilog plots for the oxidation of the isolated performic acid oxidized A and B chains of insulin. It can be seen from these that there is a "fast" tyrosyl residue in the B chain. The rate constants are: for oxidized A chain, 4.3×10^{-3} min^{-1} and for oxidized B chain, 2.0×10^{-3} and 2.6×10^{-2} min^{-1}.

Table I shows the data obtained from amino acid analysis of the control and tyrosinase-treated intact B chains at two different reaction times and the amino acid analysis of the heptapeptide obtained in these experiments. Amino acid analyses were run usually only on the long column for acidic and neutral amino acids, although periodic analyses of the basic amino acids showed no changes. For the shorter reaction time (18 min) spectral analyses gave a value of 0.37 mole of tyrosine oxidized. Amino acid analysis of the oxidized intact B chain showed that 0.35 mole of tyrosine was lost. Analysis of the heptapeptide revealed that 0.37 mole of tyrosine was lost from this fragment. Similar data are reported for a longer reaction period (30 min). From spectral data it was observed that 0.82 mole of tyrosine was oxidized. Amino acid analysis of the intact chain showed that 0.81 mole of tyrosine was lost and 0.58 mole of tyrosine was lost from the heptapeptide.

Figure 4 shows the difference spectra of trypsin-treated performic acid oxidized B chain *vs.* oxidized

B chain and trypsin. The difference spectra is very similar to the spectra observed by Laskowski *et al.* (1956) for trypsin-treated insulin.

Discussion

The kinetic data obtained and summarized in Table II show that all four of the tyrosyl groups of zinc-insulin are oxidized at the same rate, whereas in amorphous insulin, one of the four tyrosyl groups reacts four times faster than the other three. The difference in the reactivity of the tyrosyl residues of amorphous insulin when compared to that of zinc-insulin can be attributed to differences in conformation imposed by the presence of zinc in the insulin molecule.

TABLE II: Pseudo-First-Order Rate Constants for Insulin Oxidation with Tyrosinase.

Protein or Polypeptide	Pseudo-First-Order Rate Constants ($min^{-1} \times 10^3$)
Zinc-insulin	
tyrosine residues (4)	2.4 ± 0.2
Amorphous insulin	
1 tyrosine residue (Tyr-B-26)	12.0 ± 4.0
3 tyrosine residues	3.2 ± 0.3
A chain	
2 tyrosine residues	4.3 ± 0.1
B chain	
1 tyrosine residue (Tyr-26)	26.0 ± 5.0
1 tyrosine residue (Tyr-16)	2.0 ± 0.3

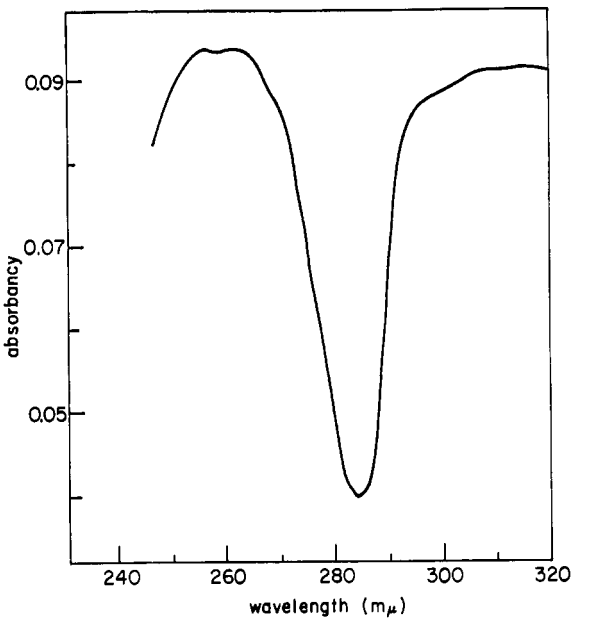

FIGURE 4: Difference spectra of performic acid oxidized B chain subjected to tryptic hydrolysis *vs.* oxidized B chain and typsin in separate cuvets. Oxidized B-chain concentration was 4 mg/ml in phosphate buffer, pH 7.4, 0.05 M.

Springell (1962), in a study of the iodination of insulin throughout the pH range 1–10, reported that the A chain of insulin is iodinated more rapidly than the B chain in intact insulin and that one tyrosyl group in the B chain is less reactive to iodination, presumably tyrosine-16. Herskovits (1965) reported, on the basis of data obtained with the solvent perturbation technique, that two of the tyrosyl residues of the insulin molecule are "buried." Aoyama *et al.* (1964) have reported that only one tyrosine group in each of the A and B chains of insulin are reactive to cyanuric fluoride. The reactive tyrosine groups were found to be A-19 and B-16 residues. Riordan *et al.* (1965) have shown that all four of the tyrosine groups of insulin are acetylated by *N*-acetylimidazole. Since the nature of the modifying reagents is so vastly different in each case, it is not surprising that the results of all these studies are not in agreement. In the case of ribonuclease, it appears from titration data that there are three abnormally ionizing groups, whereas iodination studies reveal two reactive tyrosyl groups (Cha and Scheraga, 1963a,b). Cha and Scheraga conclude that the term "buried tyrosine" means something different for each method employed.

The tyrosyl groups of the oxidized A chain react at the same rate, while one of the tyrosyl groups of oxidized B chain reacts 13 times faster than the other tyrosine. The heptapeptide, containing tyrosine-26, can be readily separated by the procedure of Sanger and Tuppy (1951) using trypsin hydrolysis and electrophoresis. It is found that the "fast" tyrosyl groups is tyrosine-26 since amino acid analysis of the isolated heptapeptide shows the loss of the oxidized group (Table I).

Calculations of the amount of each tyrosyl residue were made based on the experimentally determined rate constants and the amino acid analysis of the heptapeptide, according to eq 1. (Tyr_1) and (Tyr_2) are the concentrations of tyrosine-26 and tyrosine-16, respectively, remaining at time t. Calculated values for the times 18 and 30 min, are obtained which agree within 8 and 20%, respectively, of the observed values.

$$\frac{(Tyr_1)_t + (Tyr_2)_t}{(Tyr_1)_0 + (Tyr_2)_0} = \frac{(Tyr_1)_t}{(Tyr_1)_0 + (Tyr_2)_0} e^{-2.6(10^{-2})t} + \frac{(Tyr_2)_t}{(Tyr_1)_0 + (Tyr_2)^0} e^{-2.0(10^{-3})t} \quad (1)$$

The rate constants for the isolated oxidized A and B chains, although slightly higher in some cases, are similar to the constants for the intact insulin molecule.

Laskowski *et al.* (1956) have reported that tyrosine-26 of the B chain of intact insulin is hydrogen bonded to some unknown carboxylate ion, and this hydrogen bond is ruptured by trypsin hydrolysis. The isolated B chain when subjected to tryptic hydrolysis, gives a difference spectrum with a peak at 285 mμ (Figure 4). A similar difference spectrum is seen when the intact insulin molecule is treated with trypsin. This suggests that the performic acid oxidized B chain retains sufficient secondary or tertiary structure to allow a valid extrapolation from the isolated B chain to the intact insulin molecule.

However, we cannot exclude the possibility that the environment of the tryosine groups in amorphous insulin and in performic acid oxidized B chain is different. Since the tyrosine residues in amorphous insulin show only a fourfold difference in reaction rates with tyrosinase, and since there are three times as many "slow-reacting" tyrosines as "fast" tyrosine residues, it seems unlikely that the "fast-reacting" tyrosine group in amorphous insulin could be experimentally determined by the same technique described for the oxidized B chain.

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