Iodination of the Normal and Buried Tyrosyl Residues of Lysozyme. II. Spectrophotometric Analysis*

J. Wolff and I. Covelli†

ABSTRACT: Spectrophotometric titration of lysozyme iodinated under a variety of conditions yielded values for mono- and diiodotyrosyl residues in excellent agreement with those obtained on the same materials after hydrolysis and chromatography. Two tyrosyl residues are iodinated in water; the third one only in 8 M urea. The pK values of the iodotyrosyl residues are lower than in the free iodoamino acids. Iodination in H_2O abolished the time dependence of ionization of the buried tyrosyl residue. Although oxidation of H_2O_2 produced a similar effect, the change following iodination occurred at concentrations where nearly all the I_2 was consumed in substitution reactions. Tryptophanyl residues began to be oxidized only when >2 moles of I_2/mole of protein was used. In water at 24° this amounted to ~1 residue/mole.

In 8 M urea a second residue was oxidized. It is proposed that the third tyrosyl residue can be iodinated only under conditions during which a second tryptophanyl residue is oxidized.

In the preceding paper two of the three tyrosyl (and the histidyl) residues of lysozyme were found to be readily iodinated (Covelli and Wolff, 1966). The third tyrosyl group required 8 M urea to become available for iodination. Since Edelhoch (1962) had presented evidence that the monoiodotyrosine and diiodotyrosine of thyroglobulin could readily be detected by spectrophotometric titration, this method was applied to preparations of iodinated lysozyme. The chromatographic and specific activity data of the first paper could thus also act as a check on the spectrophotometric method not heretofore supplied. In addition, changes in the properties of the buried tyrosyl residue of partially iodinated lysozyme and the oxidation of some of the tryptophanyl residues have been investigated.

Methods

Lysozyme was iodinated, reduced, and dialyzed as described (Covelli and Wolff, 1966). Samples were diluted with 0.10 M KCl and 0.10 M phosphate buffer, pH 3.75. The absorbancies at 290, 295, 305, and 325 and occasionally at 350 and 360 mμ[1] were determined in the Beckman Model DU spectrophotometer upon addition of 2 M KOH with an Agla micrometer syringe (Edelhoch, 1962; Covelli and Wolff, in preparation). The pH was measured in the cuvet with Leeds and Northrup miniature electrodes connected with a Radiometer Model TTT-1 expanded-scale pH meter while the solution was magnetically stirred. Volume corrections for added KOH were applied where pertinent. Amounts of the various tyrosyl residues were calculated from changes in optical density with pH at 290, 295, 305, and 325 mμ assuming that the change in molar extinction coefficients due to ionization of the free amino acids is: 2100, 2400, 4000, and 4100 for tyrosine (at 290 and 295 mμ), MIT[2] (3-monoiodo-L-tyros(ine)(yl)) and DIT (3,5-diiodo-L-tyros(ine)(yl)), respectively (Edelhoch, 1962; Covelli and Wolff, in preparation). The pH range used for ΔOD calculations was >9.3 for tyrosine, 7.1–9.3 for MIT, and 4.8–7.1 for DIT. The total number of tyrosyl residues thus accounted for was about 95% of theoretical. Difference spectra were recorded with a Cary Model 14 spectrophotometer, at room temperature. Samples iodinated in water for 3 min were used to study the time dependence of ionization. They were centrifuged for 3 min at 19,000g and the clear supernatant solution was analyzed by difference spectra at 295 mμ as a function of time. Oxidation of lysozyme with H_2O_2 in 10% dioxane–bicarbonate buffer was carried out according to Hachimori *et al.* (1964) followed by dialysis.

Tryptophan oxidation was determined by color development at 330 mμ at a pH of 2.9–3.0. In preliminary experiments the model substance *N*-acetyltryptophanamide (obtained from the Cyclo Chemical Corp.) was oxidized with 1–6 moles of I_2/mole of indole at pH 8.5 in 0.2 M Tris buffer. Subsequently any excess I_2 was reduced with $K_2S_2O_3$. At pH 2.9–3.0 the molar

* From the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Maryland. *Received October 13, 1965.*

† Recipient of a NATO Fellowship. On leave from the Istituto di Patologia Generale, Università di Napoli, Italy.

[1] Although no thyroxine was found in these preparations by chromatography there was significant absorption at 350 and 360 mμ some of which titrates in the pK range of thyroxine. The importance of this color will be discussed elsewhere (Covelli, I., and Wolff, J., manuscript in preparation).

[2] The following abbreviations are used: MIT, 3-monoiodo-L-tyros(ine)(yl); DIT, 3,5-diiodo-L-tyros(ine)(yl); MIH, 2- or 5-monoiodo-L-histid(ine)(yl); DIH, 2,5-diiodo-L-histid(ine)(yl).

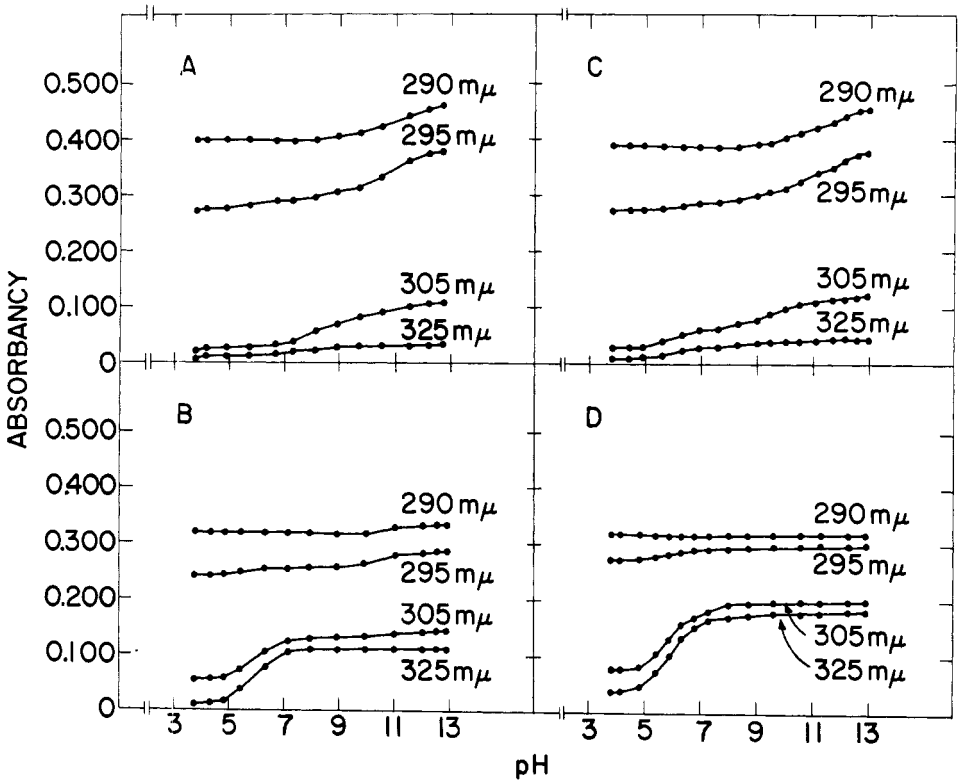

FIGURE 1: Spectrophotometric titrations at 290, 295, 305, and 325 mμ of lysozyme iodinated at 24°. A and B: iodination in 0.2 M Tris–HCl buffer, pH 8.50. C and D: iodination in 8 M urea, and 0.2 M Tris–HCl, pH 8.50. A and C: iodination with 0.9 mole of I_2/mole of protein. B and D: iodination with 12.0 moles of I_2/mole of protein. Cuvets contained 2.0 ml of 0.1 M KCl, 0.2 ml of 0.1 N HCl, 0.3 ml of 0.1 M PO_4^-, pH 3.75, and 0.5 ml of lysozyme solution. Lysozyme concentration = 1.1×10^{-5} M.

extinction coefficients of the oxidation products were: 1550, 1610, 1730 for the peptide oxidized with 1, 2, or 3 moles of I_2, respectively. No further increase in absorbancy occurred with amounts of I_2 increasing up to 6 moles/μmole of peptide. Chromatographic checks with ^{131}I-labeled I_2 showed that no substitution product had formed.

At pH 2.9–3.0 the following compounds were found not to absorb at 330 mμ: MIT, DIT, *N*-acetyl-L-diiodotyrosylglycine (kindly supplied by Dr. R. Pitt-Rivers), or *N*-acetyltryptophanamide. Of potential oxidation products neither cysteic acid nor methionine sulfone absorbs. Oxidation of *N*-acetylmethionine or *N*-acetylmethionineamide (Cyclo Chemical Corp.) with I_2 at a molar ratio of 12 (as described for *N*-acetyltryptophanamide) yielded no products absorbing at 330 mμ at pH 3.0. Although free histidine forms a variety of oxidation products with I_2 that absorb at 330 mμ, no such products were formed when *N*-benzoyl-L-histidine or *N*-benzoyl-L-histidine methyl ester were oxidized with I_2 at a molar ratio of 9. Iodination of ribonuclease A (which contains no tryptophanyl residues) with 8 moles of I_2/mole of protein at pH 8.5 in urea did not produce absorption at 330 mμ at pH 3.0.

It therefore seemed reasonable to assume that most of the extinction measured under these conditions was, in fact, due to oxidation products of tryptophanyl residues. Since there is no knowledge of the oxidation products, we have arbitrarily chosen a mean extinction coefficient of 1610 for the oxidation products. The error, thus introduced, is <7.0%. We have also assumed that these considerations apply to the tryptophanyl residues within lysozyme. This does not necessarily mean, however, that they are applicable to other proteins.

Results and Discussion

Spectrophotometric Titration. Examples of titration curves of lysozyme iodinated under a variety of conditions are shown in Figure 1. Tyrosine was read at 290 as well as 295 mμ because of an interference by MIT at 295 mμ. In free MIT this amounts to ~9% at pH 9.5 but is probably less in the iodinated lysozyme because of a shift in the phenolic dissociation of MIT toward the acid (see below). At 290 mμ, the curve was very nearly flat until a pH of 9–9.5 had been attained. The iodinated histidines have only a negligible absorption at 290 mμ or above and, hence, have been disregarded in the titrations. The degree and nature of

TABLE I: Comparison of the Yields of MIT and DIT Obtained by Chromatography and Spectrophotometric Titration.

	Moles of I_2 Added/ Mole of Lysozyme	Moles of I_2 Bound/ Mole of Lysozyme	By Chromatography			By Spectrotitration		
			MIT Residues	DIT Residues	Recovery[a]	MIT Residues	DIT Residues	Recovery[a]
H_2O[b]	0.90	0.89	0.55	0.16	0.87	0.53	0.19	0.91
	2.70	2.65	0.46	1.01	2.48	0.31	1.01	2.33
	4.80	3.90	0.20	1.64	3.48	0.16	1.62	3.40
	12.00	4.90	0.17	1.92	4.01	0.18	1.80	3.78
8 M Urea[b]	1.25	1.22	0.50	0.36	1.22	0.46	0.38	1.22
	2.70	2.65	0.53	0.98	2.49	0.42	1.01	2.44
	4.80	4.70	0.38	1.95	4.28	0.35	2.00	4.35
	12.00	6.90	0.14	2.30	5.94	0.20	2.79	5.78

[a] Moles of I_2 recovered as iodotyrosines. [b] Iodinations at +24°, pH 8.50.

substitution was checked by the chromatographic methods discussed above.

As expected from the chromatographic results (Covelli and Wolff, 1966) low levels of iodination (Figures 1A and 1C) at 24° yield only small increases in absorbancy at 305 and 325 at the respective pH zones for MIT (7.1–9.3) and DIT (4.8–7.1), respectively. Uniodinated tyrosine is still readily titratable. When the degree of iodination was high (in 8 M urea) so that the levels of organic iodine formed reached plateau values, no further tyrosines were titrated (Figure 1D), whereas in protein iodinated in H_2O, nearly a whole tyrosyl residue persists (Figure 1B).

The close correlation in the MIT and DIT values calculated from the specific activities after hydrolysis and chromatography (Covelli and Wolff, 1966) with values obtained from spectrophotometric titrations is emphasized in Table I. Over the whole range of molar ratios of [I_2]/[lysozyme] there was close agreement between the two methods. In addition, when comparisons are made on the basis of the fraction of added I_2 recovered (assuming 1 mole of organic iodine/mole of I_2 = 100%), excellent correlation was observed. This is verification, by an independent method, of the spectrophotometric titration for MIT and DIT and confirms the value of Edelhoch's method. It is also apparent from Table I that the total organic iodine recovered exceeds that which can be accounted for as MIT and DIT. It was pointed out in the previous paper that iodohistidine accounted for this difference. Obviously the iodine recovered by chemical analysis should check with that calculated from spectrophotometric titration only when there are no iodohistidines.

These data also show a marked shift toward the acid region in the midpoint of the DIT titration curve and a smaller shift for MIT. Apparent pK values (used here as the uncorrected midpoint of the titration curves) of several iodinated proteins are compared with the pK values of the free amino acids in Table II. These values are not corrected for the electrostatic effect and demonstrate that the shifts in apparent pK are qualitatively those that would be expected from the isoelectric points.

Inada (1961) has shown that one of the tyrosyl residues of lysozyme is extremely abnormal, titrating with a marked time dependence even in 8 M urea. The apparent pK in the altered protein was 12.8. Since iodination with I_2 under these conditions occurs primarily on the phenolate ion, the difference in apparent pK values above would decrease the rate of iodination of this buried tyrosyl residue by more than a factor of 100. Attempts were made to determine experimentally whether this residue is the same as that resistant to iodination in water.

The delay in ionization of the buried tyrosyl residue of native lysozyme in strong alkali was completely abolished by iodination of the other tyrosyl residues. In Figure 2 the data are plotted according to Inada (1961) where f is the fraction of un-ionized tyrosyl

TABLE II: Apparent pK Values of Tyr, MIT, and DIT in Thyroglobulin, Insulin, Lysozyme, and the Free State.

Compound	Isoelectric Point	Apparent pK[a]		
		TYR	MIT	DIT
Free amino acid[b]	...	10.13	8.2	6.36
Thyroglobulin[b]	4.7	11.35	~9.3	~7.5
Insulin[c]	5.3	...	...	7.9[d]
Lysozyme	11.1	10.5–12.8	7.9	6.0

[a] As used here apparent pK refers to the midpoint of titration curves. [b] Edelhoch, 1962. [c] Gruen *et al.*, 1959. [d] 7.2 in 0.3 M KCl.

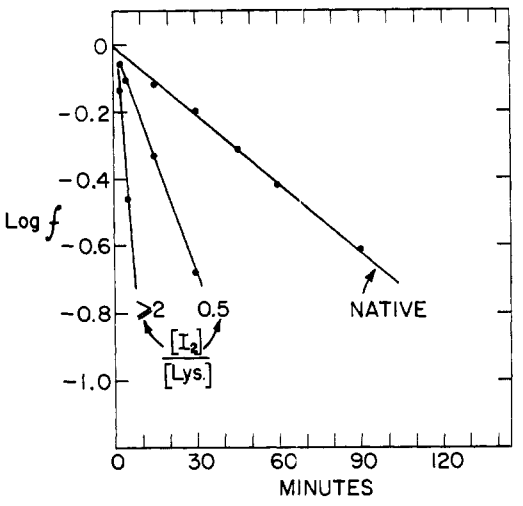

FIGURE 2: Rate of disappearance of the buried tyrosine of lysozyme iodinated in H_2O at 24°. The fraction (f) of the un-ionized tyrosyl residues remaining after addition of KOH to 1.0 M is plotted as a function of time and compared with native lysozyme. The fraction f was calculated on the basis of the ΔOD_{295} between pH 8.50 and pH 13.0 (1.0 M KOH) as a function of time.

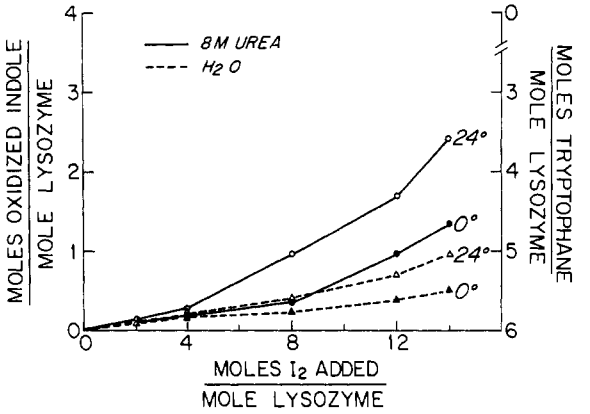

FIGURE 3: Appearance of iodination products of tryptophan in lysozyme iodinated under several conditions. The moles of oxidized tryptophan have been calculated on the basis of the OD at 330 mμ and at pH ~3.0 using $E_{330} = 1610$ (see Methods).

residues remaining (after exposure to 1.0 M KOH). When two tyrosines are fully iodinated, ionization of the third is almost instantaneous. This is true also after iodination of only the first tyrosyl residue at 24° (2 moles of I_2/mole of lysozyme). At lesser degrees of iodination the rates of ionization are intermediate between native and extensively iodinated lysozyme (Figure 2). At 0° more I_2 was required to bring about the same changes. Instantaneous ionization occurred at [I_2]/[lysozyme] ratios of ~6, whereas at a ratio of 2 intermediate rates were observed. The partial persistence of the time dependence upon partial iodination shows only that the first tyrosine iodinated must be one of the normal residues. The second tyrosyl group could still be the buried one. However, iodination of two tyrosyl residues in H_2O produced structural changes such that the apparent pK of the third was 11.0, less than the buried tyrosyl of the native molecule but still distinctly different from the 10.5 of the normal residues. Since the effect of iodination of the two tyrosyl residues produces only a small change in the net charge and hence the isoelectric point (Donovan *et al.*, 1961), it is improbable that the apparent pK of 11.0 resulted from a change in the electrostatic effect. It seems likely, therefore, that it is the same tyrosyl group that is unavailable for titration and iodination.

It has been assumed not infrequently that abnormal ionization is equivalent to abnormal reactivity toward I_2 in tyrosyl residues. While this appears to be so in some instances, it has also been shown that these two areas of the tyrosyl residue may be buried to a different extent. Although the question is not settled, it has been suggested that one of the abnormally ionizing residues of ribonuclease is readily iodinated, whereas the remaining two abnormal residues are not (Cha and Scheraga, 1963; Donovan, 1963). Similarly, although one tyrosyl group of insulin (B26) titrates abnormally (Laskowski *et al.*, 1960; Inada, 1961), all four react in water during extensive iodination (Gruen *et al.*, 1959; Springell, 1962). It should be pointed out, however, that under less drastic iodinating conditions the B chain is less reactive toward iodine (De Zoeten and Havinga, 1961; Springell, 1962).

Oxidation of Lysozyme. The iodine consumption data of the previous paper suggested the occurrence of oxidative side reactions during iodination when the ratio of [I_2]/[lysozyme] exceeded 2. The oxidation of tryptophanyl residues was assessed at 330 mμ at pH ~3 as described in the Methods. It can be seen in Figure 3 that in H_2O no more than one residue was oxidized even upon addition of 14 moles of I_2. In 8 M urea at 24° more than one tryptophanyl residue was oxidized when the [I_2]/[lysozyme] ratio was ~13. This occurred despite a relatively smaller excess of unreacted I_2 under these conditions. Oxidation of tryptophanyl residues was less at 0°. Nevertheless, more than one residue was oxidized at this temperature in 8 M urea when the ratio was 14.

The fact that the buried or third tyrosyl residue could be iodinated only in 8 M urea, and this with the development of considerable yellow color, suggested that an oxidation, particularly of tryptophan, accompanied this iodination. The tryptophan content of casein, thyroglobulin, and γ-globulin is known to decrease upon iodination (Roche and Michel, 1948; Koshland *et al.*, 1963). In lysozyme, one tryptophanyl residue is readily oxidized during iodination at pH ~5 (Hartdegen and Rupley, 1964). In the present study when the ratio of [I_2]/[lysozyme] exceeded 2, increasing amounts of iodine could not be accounted for as organic iodine or unreacted I_2 (Covelli and Wolff, 1966). This

loss was maximal in 8 M urea at 24°. Although one tryptophanyl residue was readily oxidized as soon as excess I_2 became available (Figure 3), even when only stoichiometric (8 moles of I_2/mole of lysozyme) amounts of iodine were present, oxidation of a second tryptophanyl residue seemed to occur before or during exposure of the third tyrosyl residue for iodination (24° in 8 M urea).

At 0° in 8 M urea there was only minimal DIT formation from the third tyrosine (Covelli and Wolff, 1966) and there was only minimal oxidation of the second tryptophanyl residue (Figure 3). Oxidation of lysozyme in 2 mM H_2O_2 according to Hachimori *et al.* (1964) did not expose the buried tyrosyl residue for iodination in H_2O at 24°.

Abolition of the time dependence of ionization of the third tyrosyl residue occurred when 2 (or more) moles of I_2/mole of lysozyme has been added in H_2O (24°). Since nearly all of the I_2 can be accounted for from substitution reactions (2 atoms of I/lysozyme molecule) it is apparent that there can be no stoichiometric I_2 consumption for oxidative side reactions. Also, little or no tryptophan was oxidized under these conditions (Figure 3). Identical changes were produced by oxidation in 2 mM H_2O_2 in dioxane–bicarbonate (Hachimori *et al.*, 1964). At this concentration the rate of ionization was as rapid as with >2 atoms of I bound/mole of lysozyme as depicted in Figure 2.

Two possible explanations thus remain to explain the change in ionization characteristics of the buried tyrosine. Either the reaction is oxidative and, if so, not stoichiometric in the case of I_2, or, more likely, both oxidation and iodotyrosyl formation can eliminate the time dependence. We have no basis for choosing between these at present.

These changes are not sufficient to make this residue available for iodination. Even with 18 moles of ICl/mole of lysozyme (at pH 8.50 in H_2O) only six I atoms were substituted. Approximately 2 atoms were accounted for as iodohistidines and I^- and 0.96 mole of tyrosine could still be titrated (unpublished observations). Thus, the third tyrosine did not appear to be attacked even by ICl. On the contrary, while 8 M urea is not a sufficiently potent denaturant to make the third tyrosyl group titratable, except after a time delay of some hours (Inada, 1961), it does induce such configurational changes as are needed to oxidize a second tryptophanyl residue as a prerequisite for iodination. From recent crystallographic data (Blake *et al.*, 1965), it seems likely that Tyr_{23} is involved in the so-called hydrophobic box. It is tempting to suggest that the tyrosyl unavailable to OH^- or I_2 is, in fact, the residue in this location. Studies are in progress on tryptic peptides of iodinated lysozyme to locate the iodinated and unavailable residues.

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