Ansevin, A. T., and Lauffer, M. A. (1959), *Nature 183*, 1601.
Ansevin, A. T., and Lauffer, M. A. (1963), *Biophys. J. 3*, 239.
Ansevin, A. T., Stevens, C. L., and Lauffer, M. A. (1964), *Biochemistry 3*, 1512.
Banerjee, K., and Lauffer, M. A. (1965), Program and Abstracts of the Biophysical Society, Abstract TD3, San Francisco, Calif., 1965.
Boedtker, H., and Simmons, H. (1958), *J. Am. Chem. Soc. 80*, 2550.
Caspar, D. L. D. (1963), *Advan. Protein Chem. 18*, 37.
Flory, P. J. (1936), *J. Am. Chem. Soc. 58*, 1877.
Fraenkel-Conrat, H. (1957), *Virology 4*, 1.
Haggis, G. H. (1965), *Virology 25*, 154.
Kramer, V. E., and Witmann, H. G. (1958), *Z. Naturforsch. 13b*, 1.
Lauffer, M. A. (1962), *in* The Molecular Basis of Neoplasia, Austin, Texas, The University of Texas M. D. Anderson Hospital and Tumor Institute, pp 180–206.
Lauffer, M. A. (1964a), *Biochemistry 3*, 731.
Lauffer, M. A. (1964b), *in* Symposium on Foods—Proteins and Their Reactions, Schultz, H. W., and Anglemier, A. F., Ed., Westport, Connecticut, Avi Publishing Co., Chapter 5.
Lauffer, M. A. (1966), *Biochemistry 5*, 1952.
Lauffer, M. A., Ansevin, A. T., Cartwright, T. E., and Brinton, C. C. (1958), *Nature 181*, 1338.
Scatchard, G., Batchelder, A. C., and Brown, A. (1946), *J. Am. Chem. Soc. 68*, 2320.
Schramm, G. (1943), *Naturwissenschaften 31*, 94.
Schramm, G., Schumacher, G., and Zillig, W. (1955), *Z. Naturforsch. 10b*, 481.
Smith, C. E. (1961), Ph.D. Dissertation, University of Pittsburgh, Pittsburgh, Pa.
Stevens, C. L., and Lauffer, M. A. (1965), *Biochemistry 4*, 31.

The Alteration in the Reactivity of the Tyrosine and Tryptophan Groups of Trypsin upon Combination with Protein Inhibitors*

Robert F. Steiner

ABSTRACT: When trypsin combines with any of several protein inhibitors, one to two tryptophan groups and two to three tyrosines are shielded from reaction with *N*-bromosuccinimide and iodine, respectively. This may reflect their presence in the zone of contact of trypsin and inhibitor, presumably in the active site vicinity.

The combination of trypsin with several protein inhibitors has been shown to result in distinct changes in a number of physical properties. In particular, an ultraviolet difference spectrum is developed, which has similar features for all inhibitors, although quantitative differences exist (Edelhoch and Steiner, 1965). The difference spectrum is developed at wavelengths characteristic of both tyrosine and tryptophan residues, suggesting that the environments of both groups are altered as a consequence of complex formation. The positive sign of the change in absorbancy is that usually associated with the shift of a chromophore from a polar to a nonpolar environment (Edelhoch and Steiner, 1965; Wetlaufer, 1962).

In the present investigation the change in reactivity of the tryptophan and tyrosine groups of trypsin accompanying the combination of the enzyme with several protein inhibitors has been examined. Of the four inhibitors studied [ovomucoid, lima bean (LBI),[1] pancreatic (PTI), and soy bean (STI)] only STI contains tryptophan groups. Thus, with the exception of STI, any change in tryptophan reactivity resulting from trypsin–inhibitor interaction must reflect changes in the environment of one or more of the four tryptophans (Walsh *et al.*, 1964) of trypsin alone. *N*-Bromosuccinimide (NBS), which has been shown to oxidize tryptophan groups selectively (Witkop, 1961; Green and Witkop, 1964), has been used as a specific reagent for this residue.

Iodination has been used to assess the reactivity of the tyrosine groups of trypsin. Trypsin contains 10 tyrosines (Walsh *et al.*, 1964). As tyrosines are also present in all four of the protein inhibitors (Laskowski and Laskowski, 1954), a change in tyrosine reactivity

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[1] Abbreviations used: LBI, lima bean inhibitor; PTI, pancreatic inhibitor; STI, soy bean inhibitor; NBS, *N*-bromosuccinimide; TAME, toluenesulfonylarginine methyl ester.

may stem from the tyrosyl groups of either trypsin or inhibitor or both.

Experimental Section

Methods. The oxidation of tryptophan groups with *N*-bromosuccinimide (NBS) was carried out in 0.2 M KOAc solution pH 5–5.2. The trypsin concentration was usually about 0.5 mg/ml. Volumes (10 μl) of 0.003 M NBS were added to each of two magnetically stirred quartz cuvets, one of which contained solvent and the other solution. The absorbancy at 280 mμ was observed as a function of NBS level. The number of tryptophan groups oxidized per trypsin molecule was computed from the relationship (Witkop, 1961; Green and Witkop, 1964)

$$\Delta n = \frac{1.3\,\Delta A_{280}}{5500 \times \text{molarity of trypsin}}$$

where ΔA_{280} is the limiting decrease in absorbancy. A correction was made for the drop in A_{280} arising from dilution due to the reagent added. The indole group of tryptophan is probably oxidized to oxindole under these conditions.

A difficulty repeatedly encountered with NBS titrations is the development of turbidity at high NBS levels. This tends to obscure the end point. In the present study it was minimized by avoiding a local excess of reagent through using a low concentration of NBS and efficient stirring. In practice an initial roughly linear drop of absorbancy with increasing NBS level is followed by a flat minimum and then by a gradual rise. The latter probably stems from side reactions (Witkop, 1961; Green and Witkop, 1964). A pH of 5 was chosen to avoid difficulties arising from dissociation of the trypsin–inhibitor complex at more acid pH values (Laskowski and Laskowski, 1954).

The iodination of the tyrosine groups was carried out in 0.1 M Tris–0.01 M $CaCl_2$, pH 8–9. To separate 2-ml aliquots of protein solution were added increasing volumes of 0.05 M I_2 in 0.1 M KI. After 5 min, 500 μl of 1 M lysine–0.2 M sodium thiosulfate, pH 9.3, was added. The concentration of diiodotyrosine was determined from the absorbancy at 325 mμ, where the absorption of tyrosine and monoiodotyrosine should be negligible. A molar extinction of 5.0×10^3 was assumed for ionized diiodotyrosine, which is the final product of the iodination of tyrosine in proteins (Edelhoch, 1962). At the pH of measurement (Wu and Scheraga, 1962; Walsh *et al.*, 1964), the diiodotyrosine groups should be completely ionized (Edelhoch, 1962).

The pH of iodination was kept relatively low to avoid possible dissociation of the complex under more alkaline conditions. The iodination of tyrosine is relatively slow and nonstoichiometric under these conditions, so that considerable molar excesses of iodine are required to attain the limiting extent of conversion.

At pH 8–9 free trypsin tends to autolyze rapidly. To minimize complications from this source, 0.01 M $CaCl_2$ was present in the buffer and titration of trypsin was carried out immediately after preparation of the solution. Parallel activity determinations showed that no significant loss of activity due to autodigestion occurred during the time required for reaction.

Measurements of trypsin activity were made using the toluenesulfonylarginine methyl ester (TAME) assay. The TAME assay kit supplied by Worthington Biochemical Corp. was employed. The rate of increase with time in absorbancy at 247 mμ was observed. The conditions of assay were 0.05 M PO_4^{2-}, pH 7.5, 10^{-3} mg/ml of trypsin, 25°.

To estimate the fraction of inactive trypsin in the commercial preparation, the mole ratio of STI required to produce complete inhibition was determined. A series of trypsin–STI mixtures of varying mole ratio were assayed and the mole ratio corresponding to complete inhibition determined by extrapolation. The STI–trypsin combination is 1:1 and stoichiometric under the conditions of assay. Concentrations of STI and trypsin were determined from the absorbancies of parent solutions at 280 mμ, using an optical factor of 0.650 for trypsin (Laskowski and Laskowski, 1954) and 1.06 for STI (Wu and Scheraga, 1962) to convert absorbancy to concentration (mg/ml). The STI used had been purified by chromatography on diethylaminoethyl cellulose, as described elsewhere (Steiner, 1966). Molecular weights of 24,000 and 21,500 were assumed for trypsin (Laskowski and Laskowski, 1954) and STI (Wu and Scheraga, 1962), respectively. Measurements of ultraviolet absorbancy were made with a Beckman Model DU spectrophotometer. The temperature was 25°.

Materials. Crystalline trypsin, STI, and PTI-trypsin compound were purchased from Worthington Biochemical Corp., as were purified preparations of PTI, LBI, and ovomucoid. The trypsin preparation used contained considerable inactive material, which probably resulted from inactivation of the enzyme in the course of purification. The fraction of inactive trypsin, as estimated from the mole ratio of chromatographically purified STI (Steiner, 1966) required to produce complete inhibition, was 26%.

All other reagents were analytical grade. Glass-redistilled water was used for the preparation of all solutions.

Results

Tryptophan Groups. About three of the four tryptophan groups of trypsin are oxidized by NBS at pH 5.2 (Table I). The number of reacting tryptophans is invariably reduced in the presence of inhibitor (Table I and Figures 1–3). The data in Table I give the number of shielded tryptophans when inhibitor is present in excess. The column headed "Δn" gives the apparent number of moles of tryptophan protected from reaction when 1 mole of trypsin combines with 1 mole of inhibitor, computed assuming the trypsin to be 100% active. The final column headed "Δn_{corr}" gives the same

TABLE I: Titration of Tryptophan Residues of Trypsin–Inhibitor Mixtures with *N*-Bromosuccinimide.

Inhibitor	Solvent (0.2 M)	pH	Wt Ratio (Inhibitor/ Trypsin) in Mixture	$n_{trypsin}$[a] ± 0.10	n_i[b] ± 0.10	Δn[c] ± 0.2	Δn_{corr}[d] ± 0.2	Assumed Value
STI	NaOAc	5.0	0.90	2.72	0.92	1.61	2.16	2
STI	NaOAc	5.2	1.35	2.86	1.16	1.70	2.30	
Ovomucoid	NaOAc	5.2	1.0	2.80	0	1.04	1.40	1
Ovomucoid	NaOAc	5.2	2.0	2.87	0	0.86	1.16	
LBI	NaOAc	5.2	1.0	2.89	0	1.87	2.53	2
LBI	NaOAc	5.2	1.4	2.71	0	1.72	2.30	
PTI[e]	NaOAc	5.2	0.25	(2.80)[f]	(0)	1.25		1
PTI[e]	NaOAc	5.2	0.25	(2.80)[f]	(0)	0.99		

[a] Number of tryptophans titrated/mole for trypsin in absence of inhibitor under cited conditions. [b] Number of tryptophans titrated/mole of inhibitor in absence of trypsin. [c] Decrease in apparent number of tryptophans titrated/mole of trypsin in mixture, as compared with computed sum of values for separate determinations of trypsin and inhibitor. The mole ratio of inhibitor/trypsin is 1 or greater. [d] Corrected number of masked tryptophans, computed assuming trypsin 74% active. [e] Data were obtained for 1:1 PTI–trypsin compound. [f] Assumed average value for trypsin.

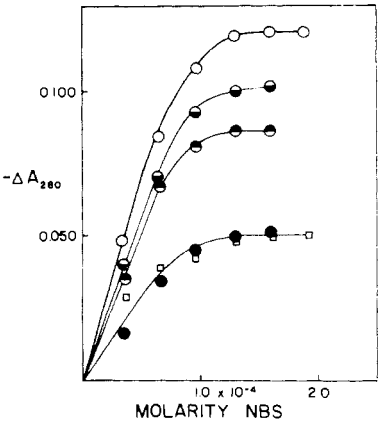

FIGURE 1: Reaction of tryptophan groups of trypsin (0.236 mg/ml) with NBS in absence and presence of varying concentrations of LBI. The solvent is 0.2 M OAc, pH 5.2. The ordinate is the decrease in absorbancy at 280 mμ; ○, no LBI; □, 0.315 mg/ml of LBI; ●, 0.095 mg/ml of LBI; ◓, 0.048 mg/ml of LBI; ◒, 0.012 mg/ml of LBI.

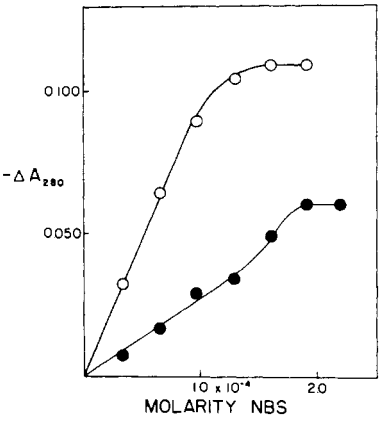

FIGURE 2: Reaction of tryptophan groups of trypsin (0.227 mg/ml) with NBS in absence (○) and presence (●) of ovomucoid (0.95 mg/ml). The solvent is 0.2 M OAc, pH 5.2. The ordinate is the decrease in absorbancy at 280 mμ.

quantity corrected for the presence of inactive trypsin ($\Delta n_{corr} = \Delta n/0.74$), assuming that only active trypsin combines with inhibitor (Laskowski and Laskowski, 1954). This correction was not made in the case of the PTI–trypsin compound.

In the cases of LBI, PTI, and ovomucoid, which contain no tryptophans, NBS treatment has no effect upon the absorbancy of inhibitor alone. Thus the lowering of the absorbancy at 280 mμ of trypsin solutions in the presence or absence of inhibitor is a direct measure of the extent of reaction of the trypsin tryptophans alone.

As Figure 1 shows, the addition of LBI causes a progressive decrease in the maximum lowering of A_{280} in the presence of NBS. The effect approaches a limit in excess LBI. The heterogeneity and uncertain molecular weight of LBI (Laskowski and Laskowski, 1954) make a direct assessment of the stoichiometry difficult, although the limiting effect is attained in the expected range of LBI concentration, if a molecular weight of 8000 is assumed (Laskowski and Laskowski, 1954).

The uncorrected number of tryptophan groups per trypsin molecule which are shielded from oxidation by excess LBI is slightly less than two. If a correction is

TABLE II: Iodination of Tyrosine Residues of Trypsin–Inhibitor Mixtures.

Inhibitor	Solvent (M)	pH	Wt Ratio (Inhibitor/ Trypsin) in Mixture	$n_{trypsin}$[a] ± 0.2	n_i[b] ± 0.2	Δn[c] ± 0.4	Δn_{corr}[d] ± 0.4	Assumed Value
STI	0.1 M Tris–0.01 M $CaCl_2$	8.2	0.90	7.60	1.90	1.80	2.43	3
STI	0.1 M Tris–0.01 M $CaCl_2$	8.5	1.00	7.90	2.00	2.2	2.97	
STI	0.1 M Tris–0.01 M $CaCl_2$	8.9	1.00	9.40	2.40	2.4	3.24	
Ovomucoid	0.1 M Tris–0.01 M $CaCl_2$	8.1	1.00	6.90	e	1.34	1.81	2
LBI	0.1 M Tris–0.01 M $CaCl_2$	8.1	1.00	7.28	e	1.96	2.65	3
PTI[f]	0.1 M Tris–0.01 M $CaCl_2$	8.5	0.25	(7.9)	g	$\geqslant 3.3$		$\geqslant 3$
PTI[f]	0.2 M Tris–0.02 M $CaCl_2$	8.5	0.25	(7.9)	g	$\geqslant 2.6$		

[a] Apparent limiting number of tyrosines/mole of trypsin which are iodinated under cited conditions in absence of inhibitor. [b] Apparent limiting number of tyrosines/mole of inhibitor which are iodinated under cited conditions in absence of trypsin. [c] Decrease in apparent limiting number of tyrosines/mole of trypsin which are iodinated in presence of excess inhibitor. [d] Corrected number of masked tyrosines, computed assuming trypsin 74% active. [e] No value cited because of uncertain molecular weight of inhibitor. [f] Data were obtained for PTI–trypsin 1:1 complex. [g] PTI was not determined separately.

made for the fraction of inactive trypsin, which is assumed not to combine with LBI, the figure is increased to between two and three (Table I).

The degree of tryptophan shielding produced by ovomucoid is somewhat less (Figure 2 and Table I). The corrected figure is close to one tryptophan per trypsin molecule. Two tryptophans are titrated by NBS in the case of the PTI–trypsin compound (Table I), indicating that one tryptophan group of trypsin is protected, in agreement with the preliminary result of Spande and Witkop (1965).

The number of tryptophans oxidized for an STI–trypsin mixture is less than the expected sum for the two components (Figure 3 and Table I). The corrected number of shielded tryptophans is close to two. Since STI contains three tryptophans (Green and Witkop, 1964; Steiner, 1966), the protected tryptophans cannot be assigned unequivocally to either component. However, since only one tryptophan of STI is titrated by NBS at pH 5 (Steiner, 1966), it is likely that at least one of the shielded tryptophans occurs in trypsin.

The principal potential sources of error in the determination of Δn, in addition to those inherent in the NBS titration (see Methods), are the autolysis of trypsin, which may result in a change in the number of reactive tryptophans, and a possible conformational change in trypsin resulting from oxidation of one or more tryptophans, which may result in partial dissocia-

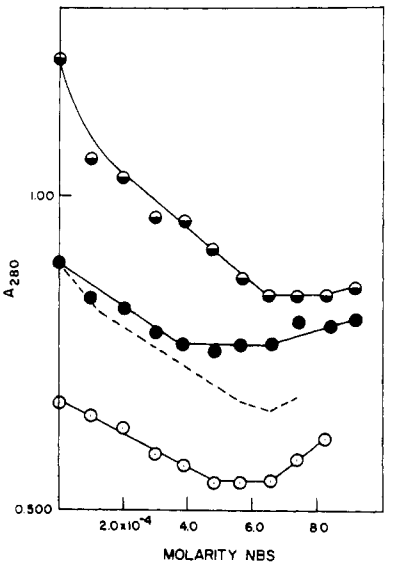

FIGURE 3: Reaction of tryptophan groups of trypsin and STI and of a trypsin–STI mixture with NBS. The solvent is 0.2 M OAc, pH 5.2. The ordinate is the absorbancy at 280 mμ. The dashed line (– – – –) represents the computed absorbancies for the mixture if the NBS reaction were uninfluenced by the trypsin–STI interaction; (◒), trypsin (0.793 mg/ml); (○), STI (0.710 mg/ml); (●), 1:1 mixture of above.

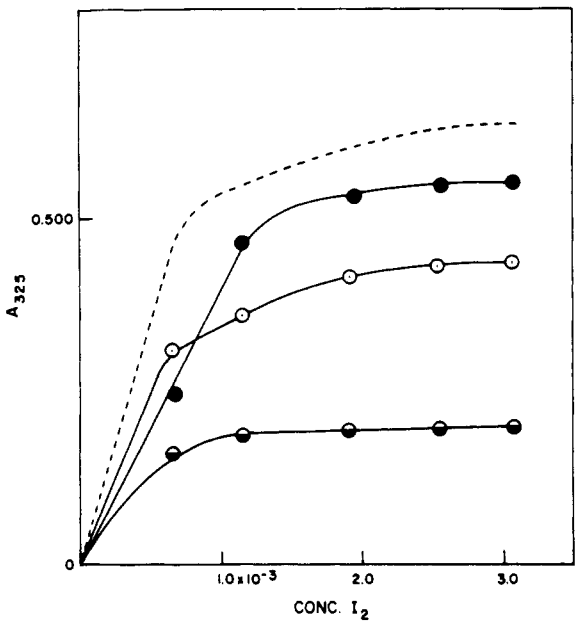

FIGURE 4: Reaction of tyrosine groups of trypsin and ovomucoid and of a trypsin–ovomucoid mixture with I_2 to form diiodotyrosine. The solvent is 0.1 M Tris–0.01 M $CaCl_2$, pH 8.13; O, trypsin (0.477 mg/ml); ⊖, ovomucoid (0.082 mg/ml); ●, 1:1 mixture of above (same concentrations); – – – –, computed curve for noninteracting mixture.

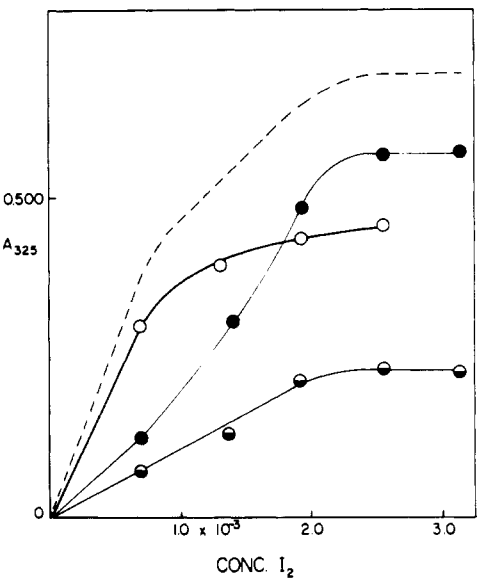

FIGURE 5: Reaction of tyrosine groups of trypsin and of LBI and of a trypsin–LBI mixture with I_2 to form diiodotyrosine. The ordinate is the absorbancy at 325 mμ. The solvent is 0.1 M Tris–0.01 M $CaCl_2$, pH 8.13; O, trypsin (0.439 mg/ml); ⊖, LBI (0.500 mg/ml); ●, 1:1 mixture of above (same concentrations); – – – –, computed curve for noninteracting mixture.

tion of the complex or alter the reactivity of other tryptophans. The first of these was probably not important under the conditions used here, since no change in trypsin activity, as assayed by TAME hydrolysis, occurred during the time required for a titration. The second factor is more difficult to assess and may introduce errors into the values for Δn.

Iodination. The limiting extent of iodination of the 10 tyrosine groups of trypsin depends somewhat upon pH (Table II), increasing appreciably between pH 8 and 9 at comparable iodine levels. The apparent limiting extents of formation of diiodotyrosine are approached asymptotically (Figures 4–6). It is of course possible that higher degrees of iodination are attainable under more drastic conditions, such as higher temperature or alkaline pH.

In the presence of excess protein inhibitor the degree of iodination is reduced considerably from the expected sum for free trypsin and inhibitor (Table II). The corrected figures for the number of shielded groups are about two for ovomucoid and about three for the other inhibitors. The quantities "Δn" and "Δn_{corr}" have a significance analogous to that of Table I.

Since all four inhibitors contain tyrosine, the masked tyrosines could occur on either component, or both. However, in the case of the PTI–trypsin compound, about three fewer groups/mole were iodinated than for trypsin itself under the same conditions, indicating that at least this number of trypsin tyrosines was shielded. It is of course likely that trypsin tyrosines are masked by combination with the other inhibitors as well.

A further effort was made to resolve the question by iodinating the complex exhaustively, separating the PTI and trypsin components by molecular sieve chromatography, on Sephadex G-100 at pH 3.0, where the complex is dissociated, and further iodinating the trypsin component. From two to three additional groups were found to be iodinated at pH 8.5. However, the activity of the trypsin was quite low, about 1/3 that of native trypsin. In view of the possibility that the inactive trypsin produced during iodination and acid treatment may have an enhanced susceptibility to iodination, this result is not conclusive, although consistent with the presence of shielded tyrosines on trypsin itself.

Discussion

From the preceding it is clear that the alteration in environment of the tyrosine and tryptophan groups of trypsin resulting from combination with protein inhibitors, which has been deduced from changes in the ultraviolet absorption spectrum and fluorescence intensity (Edelhoch and Steiner, 1965), is reflected by important changes in the susceptibility of these groups to chemical reaction. The reactivity of a tryptophan residue for NBS, or of a tyrosine group for iodine, is unquestionably dependent upon several poorly understood factors (Witkop, 1961, Edelhoch, 1962). Thus for many proteins the number of tryptophan residues

oxidized by NBS is maximal at about pH 4 and decreases at higher pH, although there is no evidence for any conformational change (Witkop, 1961).

In view of the incompletely specific character of NBS, which may react to some extent with tyrosine, histidine, or methionine under certain conditions (Witkop, 1961, Green and Witkop, 1964), it is worthwhile to consider briefly the possibility that an enhanced reactivity of one or more of these residues might produce an artificial plateau in the NBS titration curve, followed by a drop in A_{280} at higher NBS levels. In parallel experiments upon trypsin in the absence and presence of each of the protein inhibitors no subsequent drop in A_{280} after the first plateau was found at [NBS]/[trypsin] ratios up to 50:1, but only a gradual rise. Hence it is unlikely that this is a complicating factor in these experiments. Moreover, under appropriate conditions all of the tryptophans of trypsin and of STI can be titrated with NBS, with no sign of any secondary plateau (Green and Witkop, 1964; Steiner, 1966).

With regard to the iodination reaction, since iodine may react with histidine (Glazer and Sanger, 1964) it is desirable to examine the possibility that this reaction might contribute to the absorbance at 325 mμ and affect the values for diiodotyrosine. The use of spectral data to estimate the iodotyrosine content of a protein has been carefully examined in the case of thyroglobulin of varying degrees of iodination (Edelhoch, 1962). The self-consistency of the results, as established by a variety of internal checks, indicates that any contribution to the absorbancy above 300 mμ arising from alteration of histidine is insufficient to affect the results appreciably. Preliminary results indicate that the products formed by the treatment of histidine with a large excess (mole ratio, 100:1) of iodine under the conditions used in these studies had a molar absorbancy <900 at 325 mμ (R. F. Steiner, unpublished data). Since we are concerned here only with differences in the number of tyrosines reacting, it is unlikely that important errors arise from this source, although a minor quantitative distortion may be present.

The change in reactivity of tyrosine and tryptophan could be explained in either of two ways. (a) The alteration in environment results from the proximity of the group to the zone of contact of the trypsin and inhibitor molecules and reflects either an envelopment of the group by the inhibitor, which renders it sterically inaccessible to reagent, or a modification of its properties through the proximity of one or more groups on the inhibitor surface. (b) The affected groups are not located in the zone of contact but are affected indirectly by a conformational change which results from the combination of trypsin with inhibitor.

A final choice between these alternatives must of course await more definitive information as to the conformation of trypsin. However, at the present time the first explanation appears somewhat more plausible. While minor alterations in optical rotation and other physical properties suggest that a conformational change may occur for trypsin or inhibitor, or both (Edelhoch and Steiner, 1965), the changes are rather slight to reflect the major structural transition required to account for the effects reported in this paper. If (a) is indeed the correct explanation, then the positive difference spectrum (red shift) developed in the wavelengths characteristic of tryptophan and tyrosine (Edelhoch and Steiner, 1965) is consistent with an actual envelopment of these groups upon interaction.

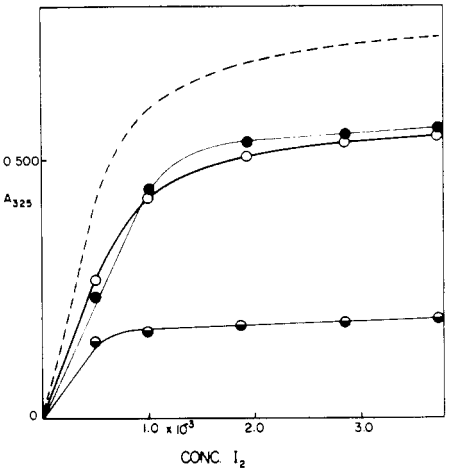

FIGURE 6: Reaction of tyrosine groups of trypsin and of STI and of a trypsin–STI mixture with I_2 to form diiodotyrosine. The ordinate is the absorbancy at 325 mμ. The solvent is 0.1 M Tris–0.01 M $CaCl_2$, pH 8.48; ○, trypsin (0.486 mg/ml); ◒, STI (0.485 mg/ml); ●, 1:1 mixture of above (same concentrations); – – – –, computed curve for noninteracting mixture.

The presence of tyrosine and tryptophan groups in the zone of contact of trypsin and inhibitor does not of course necessarily indicate that these residues form part of the active site of trypsin, but does suggest that they lie near it. Identification of the shielded groups within the known amino acid sequence of trypsin would yield valuable clues as to the conformation of trypsin. Work on this aspect is continuing.

References

Edelhoch, H. (1962), *J. Biol. Chem. 237*, 2778.

Edelhoch, H., and Steiner, R. F. (1965), *J. Biol. Chem. 240*, 2877.

Glazer, A. N., and Sanger, F. (1964), *Biochem. J. 90*, 92.

Green, N. M., and Witkop, B. (1964), *Trans. N. Y. Acad. Sci. 26*, 659.

Laskowski, M., and Laskowski, M., Jr. (1954), *Advan. Protein Chem. 9*, 203.

Spande, T. F., and Witkop, B. (1965), *Biochem. Biophys. Res. Commun. 21*, 131.

Steiner, R. F. (1966), *Arch. Biochem. Biophys.* (in press).
Walsh, K. A., Kauffman, D. L., Kumar, K. S. V., and Neurath, H. (1964), *Proc. Natl. Acad. Sci. U. S. 51*, 301.
Wetlaufer, D. B. (1962), *Advan. Protein Chem. 17*, 303.
Witkop, B. (1961), *Advan. Protein Chem. 16*, 221.
Wu, Y., and Scheraga, H. (1962), *Biochemistry 1*, 905.

Inter- and Intramolecular Interactions of α-Lactalbumin. VI. Optical Rotation Dispersion Properties*

Martin J. Kronman, Robert Blum, and Leo G. Holmes

ABSTRACT: Acid denaturation of α-lactalbumin occurring below pH 4 is accompanied by a decrease in the value of the optical rotation dispersion parameter, b_0, of the order of 75°. Moffitt–Yang plots of the native protein (*ca.* pH 6) in the visible and near-ultraviolet regions exhibited systematic deviations at the lower wavelengths (Hg lines 334 and 313 mμ). Plots for the acid-denatured protein were linear for the entire wavelength range considered. The amplitudes of troughs of the 225-mμ Cotton effects were virtually identical for the pH 2 and 6 protein, indicating that the change in b_0 was not due to "melting out" of helical regions of the α-lactalbumin molecule. Measurement of rotations in the range 250–300 mμ revealed a system of Cotton effects for the native protein (pH 6 and 7.48) with peaks at *ca.* 300 and 280–290 mμ, a trough at 290–295 mμ, and a broad plateau region extending from *ca.* 255 to 275 mμ. The rotatory dispersion spectrum for the acid-denatured protein was devoid of such Cotton effects. The alkaline-denatured protein (pH 11.4) showed a smaller decrease in b_0 with the Cotton effects system (250–300 mμ) being still evident but significantly reduced in amplitude. The Cotton effects observed for the native protein are probably due to the three "buried" tryptophans which are frozen in particularly favorable conformations.

Denaturation, which involves swelling of the α-lactalbumin molecule, permits greater free rotation of these tryptophans, thereby eliminating these special conformations. Since alkaline denaturation involves a lesser degree of swelling, these Cotton effects persist to some degree. Both the changes in b_0 and the systematic deviations in the Moffitt–Yang plots appear to be a consequence of the side chain Cotton effects. These observations illustrate the caution that must be exercised in interpreting optical rotation dispersion measurements for proteins with high contents of aromatic amino acids.

In this series of papers we have been considering various aspects of the several conformational changes that α-lactalbumin is capable of undergoing. The most subtle of these occurs when α-lactalbumin is brought from 25 to 0–2° (Kronman and Holmes, 1965). It is seen most distinctly at pH 6 where on lowering the temperature the two "exposed" tryptophan groups become inaccessible to the larger perturbants, sucrose and glycerol, but are "seen" by the smallest perturbant, heavy water (Kronman and Holmes, 1965). The two tryptophan groups involved appear to lie in "crevices" which "contract" as the temperature is lowered. Below pH 4 a more drastic conformational change occurs which, although accompanied by a relatively large "denaturation blue shift" of the tryptophan absorption spectrum (Kronman *et al.*, 1965b), does not involve an increased "exposure" (solvent perturbation) of tryptophan groups (Kronman and Holmes, 1965). Above pH 10 a process similar to that occurring below pH 4 takes place: swelling of the molecule occurs (F. M. Robbins, R. E. Andreotti, L. G. Holmes, and M. J. Kronman, manuscript in preparation; M. J. Kronman, L. G. Holmes, and F. M. Robbins, manuscript in preparation) accompanied by a short wavelength shift of the tryptophan absorption spectrum (M. J. Kronman, L. G. Holmes, and F. M. Robbins, manuscript in preparation).

Preliminary measurements of the optical rotation of α-lactalbumin carried out in collaboration with the late L. Weil at the Eastern Regional Research Laboratory suggested that the pH 4 conformational change might involve some "melting out" of helical regions of the molecule. Subsequent determination of the optical rotation dispersion of α-lactalbumin carried out in the Natick Laboratories and tentative interpretation of the changes in the parameter b_0 had indicated that as much as one-third of the helical content had been lost.

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