# Exposure of the Tyrosyl and Tryptophyl Residues in Trypsin and Trypsinogen*

German B. Villanueva† and Theodore T. Herskovits‡

ABSTRACT: The solvent perturbation of the tyrosyl and tryptophyl residues in trypsin and trypsinogen have been examined using several perturbants ranging in diameter from 4 to 8.4 Å as probes for surface groups. In the two enzymically active $\alpha$ and $\beta$ fractions of trypsin isolated by the chromatographic procedures of Schroeder and Shaw (Schroeder, D. D., and Shaw, E. (1968), *J. Biol. Chem. 243*, 2943) 4 to 6 of the 10 tyrosyls and 2 to 3 of the 4 tryptophyl residues appear to be exposed to solvent access and the remaining groups seem to be buried. In the third, inert, fractions of the commercial enzyme preparations used nearly all of the tyrosyl and tryptophyl residues seem to be exposed. A correspondingly higher degree of exposure is found in cases of the unfractionated parent materials. In the precursor of the enzyme, trypsinogen, about the same average number of tryptophyl residues are found to be exposed. However, on an average of about 1 to 1.6 additional tyrosyls seem to be exposed in trypsinogen. The greater degree of tyrosyl burial accompanying the activation of trypsinogen, suggests changes in the chain conformation at the surface areas of the protein to a more compact structure.

Several recent studies have dealt with the chemical reactivity and environment of the tyrosyl residues in trypsin and trypsinogen (Smillie and Kay, 1961; Inada *et al.*, 1964; Riordan *et al.*, 1965; Lazdunski and Delaage, 1965; Hachimori *et al.*, 1966; Delaage *et al.*, 1968; Kenner *et al.*, 1968; Gorbunoff, 1969; Herskovits and Villanueva, 1969). These studies have indicated that of the ten tyrosyl residues four to six are rapidly ionizing and chemically reactive and therefore must be located at surface positions of the enzyme and zymogen. Much less information is available concerning the reactivity and location of the four tryptophyl residues. Hachimori *et al.* (1964) have found that two to three of the tryptophyls can be oxidized by hydrogen peroxide at neutral pH. The studies of Spande *et al.* (1966) and Delaage *et al.* (1968), on the other hand, have shown that at most one groups is reactive at pH 7 toward *N*-bromosuccinamide and the Koshland reagent 2-hydroxy-5-nitrobenzyl bromide (Koshland *et al.*, 1964). However, an increasing number of these groups is found to react with *N*-bromosuccinimide in acidic solutions. At pH 4 for example, 3.3 and 3.6 groups are reactive in trypsinogen and trypsin, and nearly all four groups are found to react in trypsin at pH 3 (Spande *et al.*, 1966).

In view of these observations concerning the behavior of the tryptophyl residues and also the desire to gain further information related to the location of the tyrosyl residues in trypsin and trypsinogen, the solvent perturbation of both proteins with various perturbants was examined. Our earlier investigations on whole typsin and trypsinogen (Herskovits and Villanueva, 1969) were extended to the two active forms of trypsin, $\alpha$- and $\beta$-trypsin, discovered by Schroeder and Shaw (1968). In addition we have also studied the properties of the third, inert chromatographic fraction of trypsin as well as those of the precursor of the enzyme, trypsinogen. This paper reports the results of these investigations.

## Experimental Procedure

*Materials.* The three commercial bovine trypsin preparations employed as starting materials in this study were purchased from Worthington and Sigma (both twice-crystallized preparations), and Novo Industries, Copenhagen (crystalline, salt-free preparation). Trypsinogen (once crystallized) was obtained from Worthington. SE-Sephadex C-50 (beaded) was obtained from Pharmacia, while the polyacrylamide gel beads (Lypogel) used for the concentration of the chromatographed enzyme fractions was purchased from Gelman Instruments Company. Benzamidine hydrochloride hydrate, *p*-toluenesulfonyl-L-arginine methyl ester hydrochloride, and *N*-$\alpha$-carbobenzoxy-L-lysine-*p*-nitrophenyl ester were obtained, respectively, from Aldrich, Calbiochem, and Cyclo Chemicals Co. The *N*-acetyl ethyl esters of tyrosine and tryptophan were Mann Research Laboratories products. All other reagents and solvents used were of the purest commercially available quality. The water used was distilled in an all-glass still.

*Chromatography.* Separation of the commercial trypsins into the three major constituents, $\alpha$-, $\beta$-, and inert trypsin was achieved by means of the chromatographic procedures developed by Schroeder and Shaw (1968). In typical experiments 1 g of enzyme dissolved in 10 ml of 0.125 M, pH 7.1, Tris-HCl, containing 0.02 M $CaCl_2$ and 0.001 M benzamidine hydrochloride was processed on a 2.5 × 80 cm SE-Sephadex (C-50) column washed and equilibrated with the same solvent system in the cold (2–4°). Fractions (9–10 ml), at flow rates of 15–20 ml per hour, were collected in tubes containing 1 ml of 1.25 M potassium formate, pH 2.9 (Schroeder and Shaw, 1968). The central fractions of the individual components were usually concentrated by a factor of about two using Lypogel (5% by weight or 5 g of solid per 100 ml of pooled enzyme), followed by dialysis in the cold against several changes of 0.001 M HCl–0.02 M $CaCl_2$ required to remove the benzamidine present. These solutions served exclusively as stock solutions from which dilutions were made for our difference spectral, optical rotatory dispersion (ORD), and circular dichroism measure-

* From the Department of Chemistry, Fordham University, Bronx, New York 10458. *Received April 19, 1971.* This investigation was supported by Grant GM 14468 from the National Institutes of Health, United States Public Health Service, and a Faculty Research Grant from Fordham University.

† Part of this work was taken from the thesis for the Ph.D. degree, Fordham University, 1972.

‡ To whom correspondence should be addressed.

TABLE I: Activity of Different Forms of Trypsin.

| Protein | Per Cent of Total Sample | Activity[a] with *p*-Toluenesulfonyl-L-arginine Methyl Ester | Estimate of Active-Site Concentration[b] with *N*-α-Cbz-L-lysine-*p*-nitrophenyl Ester | | |
|---|---|---|---|---|---|
| | | | Protein Concn (M × $10^5$) | Active-Site Concn (M × $10^5$) | Per Cent Activity |
| Commercial trypsin | 100 | 222 | 0.65 | 0.41 | 63 |
| α-Trypsin | 22–38 | 300 | 2.58 | 2.27 | 88 |
| β-Trypsin | 39–42 | 444 | 2.16 | 1.95 | 90 |
| Inert trypsin | 22–36 | 0 | 3.10 | 0 | 0 |

[a] Enzyme activity expressed in micromoles of *p*-toluenesulfonyl-L-arginine methyl ester hydrolyzed per minute per mg of enzyme. [b] Estimates based on the procedure of Bender *et al.* (1966).

ments (CD). Essentially the same procedure was used to purify and concentrate the precursor of the enzyme, trypsinogen. Unlike the chromatographic patterns of whole trypsin (Schroeder and Shaw, 1968; Sipos and Merkel, 1970), the elution patterns of trypsinogen revealed essentially no inert material and relatively small quantities of α- and β-trypsin.

Protein concentrations were based on spectrophotometric measurements, using the per cent extinction coefficients, $\epsilon_{1\,cm}^{1\%}$ 15.2, 15.0, and 14.9 at 280–282 mμ for α, β, and inert fractions of trypsin, respectively. For trypsinogen and whole trypsin the values of 15.2 (Smillie and Kay, 1961) and 15.3 (Trowbridge *et al.*, 1963) were used. The values for the three purified fractions of trypsin were based on gravimetric and spectrophotometric determinations carried out in our laboratory. Corrections for moisture content were based on drying of separate samples of the individual protein fractions at 105–107° until no detectable changes in weights were noted. The protein fractions used for these measurements were exhaustively dialyzed in the cold against frequent changes of freshly distilled water. The absence of salt (chlorine ion) in the final dialysates was tested by means of the silver nitrate test, carried out on samples of the spent dialysate solutions. The final protein solutions were then lyophilized and used as required. The concentrations of the *N*-acetyl ethyl esters of tryptophan and tyrosine model compounds were based on the molar extinction coefficients of 5550 at 282 mμ and 1340 at 274.5 mμ (Beaven and Holiday, 1952; Wetlaufer, 1962).

*Activity Measurements.* Enzymic activities and active-site titration with *p*-toluene-L-arginine methyl ester and *N*-α-carbobenzoxy-L-lysine-*p*-nitrophenyl ester of the unfractionated enzyme and the three chromatographic fractions were made using the procedures of Hummel (1959) and Bender *et al.* (1966). Table I presents a summary of the yields and activity estimates obtained on the Worthington trypsin, used for much of the work reported in this paper.

*Methods.* Difference spectral measurements were made on a Cary 14 recording spectrophotometer equipped with a 10-fold scale expander. The difference spectral methods and preparation of solutions were previously described (Herskovits and Laskowski, 1962a; Herskovits, 1967). The model data of Herskovits and Sorensen (1968a,b) were used for most of the calculations. The perturbation data required for the calculations involving 20% propylene glycol and tetramethylurea is given in Table II. Optical rotatory dispersion and circular dichroism measurements were made with a Cary 60 recording spectropolarimeter equipped with a Model 6002 circular dichroism attachment.

In order to minimize the possible effects of autolysis of the enzyme, difference spectral and other measurements were usually made within 5–10 min after dilution and mixing of the solutions and solvents. Stock solutions of the chromatographed enzyme fractions were kept in the cold, at pH 3 and in the presence of 0.02 M $CaCl_2$. For the difference spectral measurements usually twofold dilutions were made using the acidic protein stock solutions, solvent, and 40% perturbant, with the latter two containing twice the desired final quantities of buffer or salt.

## Results

*Solvent Perturbation Studies.* Figure 1 presents a comparison of some of our results obtained with three of the isolated trypsin fractions, α-, β-, and inert trypsin. Also included in this figure is the perturbation difference spectra obtained with trypsinogen purified by means of the same chromatographic procedure (Schroeder and Shaw, 1968). The perturbant used for these experiments was 20% glycerol. All of our data was analyzed using the procedures described by Herskovits and Sorensen (1968a,b). Estimates of the apparent number of

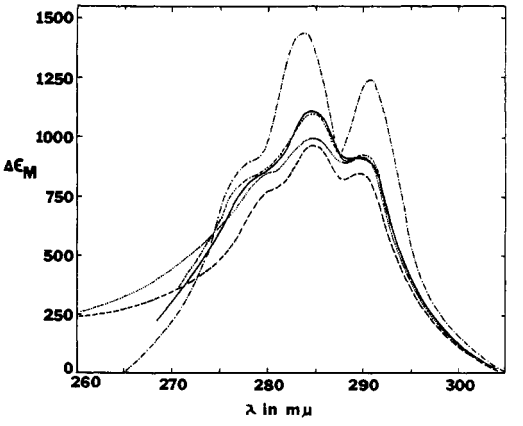


FIGURE 1: Comparison of the solvent perturbation difference spectra of the two active α and β fractions of trypsin with the third inert fraction and that of trypsinogen. The perturbant used was 20% glycerol. - - -, α-trypsin, pH 7.1, 0.1 M Tris–0.01 M $CaCl_2$; . . . . . , β-trypsin, pH 7.1, 0.1 M Tris–0.01 M $CaCl_2$; – – · · – – · · trypsinogen, pH 2.9, 0.1 M $Cl^-$–0.01 M $CaCl_2$; –·–·–, inert trypsin, pH 7.1, 0.1 M Tris–0.01 M $CaCl_2$; ———, trypsinogen, pH 7.1, 0.1 M Tris–0.01 M $CaCl_2$. Protein concentration 0.05 to 0.1%.

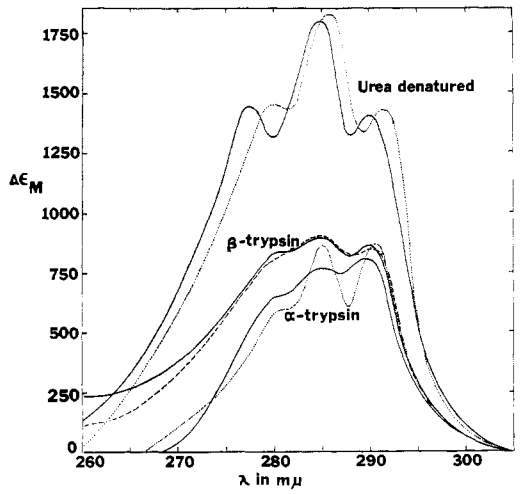


FIGURE 2: The effects of pH and urea denaturation on the solvent perturbation difference spectra of trypsin obtained with 20% ethylene glycol as perturbant. Solid lines represent the perturbation data obtained on native trypsin (in 0.1 M, pH 7.1, Tris buffer–0.01 M $CaCl_2$) and disulfide-cleaved unfractionated trypsin in 8 M urea (pH 4.0, 0.1 M $Cl^-$–0.018 M 2-mercaptoethanol). The dashed line represents the difference spectra of β-trypsin at pH 3.1 (in 0.1 M $Cl^-$–0.01 M $CaCl_2$). The dotted lines represent calculated curves giving the best fit of the data. The curve for α-trypsin was calculated on the basis of 2.7 exposed tryptophyl and 4.2 exposed tyrosyl residues, while for the denatured protein, 3.7 tryptophyl and 10 tyrosyl residues were required to give the best fit. The protein concentration used ranged from 2.3 to 2.8 $\times 10^{-5}$ M.

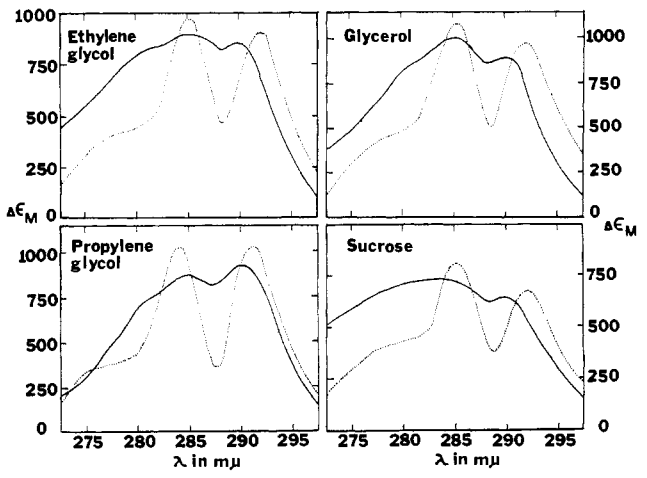


FIGURE 3: A comparison of the solvent perturbation data of β-trypsin at pH 7.1, 0.1 M Tris–0.01 M $CaCl_2$ obtained with 20% ethylene glycol, glycerol, propylene glycol, and sucrose. The dotted lines represent calculated curves based on eq 1 and the best values of the $a$ and $b$ parameters giving the closest fit of the experimental data. These values are listed in the last two columns of Table III as apparent numbers of exposed tryptophyl and tyrosyl residues. The protein concentrations used ranged from 2.6 $\times 10^{-5}$ to 3.3 $\times 10^{-5}$ M.

tryptophyl and tyrosyl residues were obtained by the best fit of the protein curves using the relationship

$$\Delta\epsilon_\lambda(\text{protein}) = a\Delta\epsilon_\lambda(\text{Trp}) + b\Delta\epsilon_\lambda(\text{Tyr}) \qquad (1)$$

where $a$ represents the number of exposed tryptophyl residues, $b$ the number of tyrosyls, $\Delta\epsilon_\lambda$(Trp) and $\Delta\epsilon_\lambda$(Tyr), respectively, the molar absorbance difference values of free tryptophan and tyrosine as a function of wavelength, λ. The tabulated model data of the *N*-acetyl ethyl esters of tryptophan and tyrosine, obtained with various perturbants were used (Herskovits and Sorensen, 1968a; and Table II). Figures 2 and 3 show some of

TABLE II: Molar Absorbance Difference Values of the *N*-Acetyl Ethyl Esters of Tyrosine and Tryptophan Obtained with 20% Propylene Glycol and Tetramethylurea as Perturbants.[a,b]

| | Ac-Tyrosine-OEt | | Ac-Tryptophan-OEt | |
|---|---|---|---|---|
| λ (mμ) | Propylene Glycol | Tetra-methylurea | Propylene Glycol | Tetra-methylurea |
| 350.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 315.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 310.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 305.0 | 0.0 | 1.3 | 4.4 | 0.0 |
| 300.0 | 0.0 | 4.1 | 37.0 | 33.0 |
| 297.5 | 0.0 | 7.8 | 73.9 | 103.6 |
| 295.0 | 1.3 | 13.8 | 143.5 | 222.3 |
| 294.0 | 2.7 | 16.8 | 178.3 | 304.7 |
| 293.0 | 5.4 | 23.2 | 230.4 | 414.2 |
| 292.5 | 8.0 | 27.0 | 252.2 | 484.2 |
| 292.0 | 9.4 | 34.5 | 282.6 | 541.3 |
| 291.5 | 12.1 | 42.0 | 295.7 | 564.7 |
| 291.0 | 14.8 | 52.8 | 304.4 | 568.5 |
| 290.0 | 21.5 | 70.0 | 265.2 | 525.9 |
| 289.0 | 30.8 | 99.5 | 169.6 | 256.2 |
| 288.5 | 38.9 | 124.6 | 108.7 | 102.1 |
| 288.0 | 45.0 | 138.2 | 52.2 | 7.2 |
| 287.0 | 60.3 | 181.3 | 30.4 | −54.3 |
| 286.0 | 75.1 | 207.1 | 87.0 | 32.2 |
| 285.5 | 85.8 | 212.6 | 126.1 | 152.0 |
| 285.0 | 91.2 | 214.4 | 152.2 | 204.1 |
| 284.0 | 92.5 | 197.2 | 191.3 | 290.2 |
| 283.0 | 84.5 | 162.7 | 191.3 | 270.8 |
| 282.0 | 53.6 | 104.1 | 160.9 | 202.8 |
| 281.5 | 42.9 | 82.9 | 139.1 | 140.6 |
| 281.0 | 34.9 | 68.4 | 121.7 | 104.7 |
| 280.0 | 26.8 | 56.0 | 100.0 | 63.0 |
| 279.0 | 33.5 | 70.5 | 87.0 | 40.9 |
| 278.5 | 38.9 | 85.0 | 78.3 | 19.5 |
| 278.0 | 41.6 | 88.4 | 69.6 | −14.0 |
| 277.0 | 48.3 | 92.2 | 52.2 | −37.5 |
| 276.0 | 40.2 | 71.1 | 52.2 | −28.5 |
| 275.0 | 26.8 | 42.2 | 69.6 | −23.1 |
| 274.0 | 13.4 | 24.4 | 73.9 | −25.4 |
| 272.5 | 0.0 | −9.8 | 56.5 | −19.4 |
| 270.0 | −8.0 | −24.6 | 17.4 | −60.8 |
| 267.5 | −13.4 | −40.0 | −4.4 | −135.0 |
| 266.0 | −16.1 | −54.0 | −13.0 | −169.0 |
| 265.0 | −20.2 | −62.0 | −13.0 | −181.0 |
| 262.5 | −16.1 | −61.0 | −26.1 | −202.0 |
| 260.0 | −13.4 | −74.0 | −43.4 | −261.0 |
| 255.0 | −10.7 | | −69.6 | |
| 250.0 | −13.4 | | −78.3 | |
| 245.0 | −17.4 | | −52.2 | |
| 242.5 | −20.2 | | −26.1 | |
| 240.0 | −24.1 | | 30.4 | |

[a] 0.1 M $Cl^-$–0.01 M, pH 6.8, phosphate buffer, 25°. [b] Data based on three to six average determinations.

TABLE III: Difference Spectral Parameters of Purified $\alpha$-, $\beta$-, and Inactive Trypsin and Trypsinogen.[a,b]

| Perturbant 20%[c] | Mean Diameter (Å) | Molar Absorptivity Difference $\Delta\epsilon_{290-292}$ | Molar Absorptivity Difference $\Delta\epsilon_{284-286}$ | Apparent Number of Exposed Residues: Tryptophyl | Apparent Number of Exposed Residues: Tyrosyl |
|---|---|---|---|---|---|
| | | | $\alpha$-Trypsin | | |
| Dimethyl sulfoxide | 4.0 | 1240 | 1255 | 2.3 | 4.6 |
| Ethylene glycol | 4.4 | 805 | 770 | 2.7 | 4.2 |
| Glycerol | 5.2 | 850 | 970 | 2.7 | 6.3 |
| Propylene glycol | 5.7 | 800 | 710 | 2.7 | 4.1 |
| Tetramethylurea | 6.3 | 1300 | 1345 | 2.2 | 4.5 |
| Hexaethylene glycol[d] | 9.2 | 1115 | 1195 | 1.6 | 6.0 |
| Sucrose | 9.4 | 550 | 685 | 2.8 | (8.8)[e] |
| | | | Average exposure | 2.4 ± 1.0 | 5.0 ± 1 |
| | | | $\beta$-Trypsin | | |
| Dimethyl sulfoxide | | 1320 | 1335 | 2.4 | 4.8 |
| Ethylene glycol | | 860 | 895 | 2.7 | 5.2 |
| Glycerol | | 910 | 1005 | 2.9 | 6.4 |
| Propylene glycol | | 920 | 880 | 3.1 | 4.4 |
| Tetramethylurea | | 1265 | 1520 | 2.1 | 5.2 |
| Hexaethylene glycol[d] | | 1170 | 1350 | 1.7 | 6.8 |
| Sucrose | | 650 | 725 | 3.2 | (9.5)[e] |
| | | | Average exposure | 2.6 ± 1.0 | 5.5 ± 1 |
| | | | Inert Trypsin | | |
| Dimethyl sulfoxide | | 2020 | 2475 | 3.4 | 9.8 |
| Ethylene glycol | | 1400 | 1780 | 4.4 | 11.0 |
| Glycerol | | 1240 | 1440 | 3.8 | 9.8 |
| Propylene glycol | | 1390 | 1730 | 4.1 | 11.1 |
| Tetramethylurea | | 2005 | 2510 | 2.9 | 9.0 |
| Hexaethylene glycol[d] | | 1945 | 2330 | 2.6 | 11.8 |
| | | | Average exposure | 3.5 ± 1.0 | 10.4 ± 1 |
| | | | Trypsinogen | | |
| Dimethyl sulfoxide | 4.0 | 1545 | 1710 | 2.9 | 6.0 |
| Ethylene glycol | 4.4 | 740 | 890 | 2.8 | 5.2 |
| Glycerol | 5.2 | 910 | 1120 | 3.0 | 7.5 |
| Propylene glycol | 5.7 | 1070 | 1170 | 3.4 | 7.9 |
| Tetramethylurea | 6.3 | 1330 | 1500 | 2.1 | 5.1 |
| Hexaethylene glycol[d] | 9.2 | 1120 | 1385 | 1.5 | 6.7 |
| Glucose | 7.2 | 360 | 445 | 2.0 | 7.3 |
| Sucrose | 9.4 | 330 | 445 | 1.9 | 7.0 |
| | | | Average exposure | 2.5 ± 1.0 | 6.6 ± 1 |

[a] Solvent, 0.1 M, pH 7.1, Tris–0.01 M $CaCl_2$. [b] The $\alpha$-trypsin parameters given represent the average values obtained on the Worthington, and Novo trypsins, the $\beta$-trypsin values represent the averages of the Worthington, Sigma, and Novo trypsins, while the values obtained on the inert fraction represent the average of the Worthington and Sigma preparations. A single preparation of trypsinogen obtained from Worthington was used. [c] With the exception of sucrose, 20 volumes of liquid perturbant was used per 100 volumes of solution. Sucrose solutions contained 21.6 g of solid per 100 ml of solution (20%, w/w). [d] Carbowax 300 (Union Carbide products). Average number of polymerization = 6. [e] Not included in the estimates of average exposures (see text concerning this).

the protein curves obtained (represented by the solid lines) and the best-fit curves based on the model compound data (represented by the dotted line). The problems attending the curve fitting and interpretation of protein and model compound data have been fully described in previous publications from this laboratory (Herskovits and Sorensen, 1968a,b). The loss and obliteration of difference spectral detail usually observed with protein curves such as those in Figures 2 and 3 have been attributed to the presence of some partially buried groups and the heterogeneity of the environment of surface groups relative to the homogeneous environment of the tryptophyl and tyrosyl model compounds in solution.

Table III presents a summary of the perturbation data of the two active $\alpha$ and $\beta$ fractions of trypsin as well as the third inert fraction and trypsinogen. The molar absorbance difference values $\Delta\epsilon_M$ at 284–286-m$\mu$ and 290–292-m$\mu$ peaks listed represent the average values of two $\alpha$-trypsin preparations (Worthington and Novo enzymes), three $\beta$-trypsins (Worthington, Novo, and Sigma), and two inert trypsins (Worthington and Sigma). The trypsinogen data given represents the

TABLE IV: Effects of pH and Urea Denaturation on the Solvent Perturbation Difference Spectral Parameters of Trypsin and Trypsinogen Obtained with 20% Ethylene Glycol.

| Solvent | pH | Molar Absorbance Difference | | Apparent Number of Exposed Residues | |
|---|---|---|---|---|---|
| | | $\Delta\epsilon_{290-292}$ | $\Delta\epsilon_{284-286}$ | Tryptophyl | Tyrosyl |
| | | Trypsinogen | | | |
| 0.1 M $Cl^-$–0.01 M phosphate | 6.8 | 800 | 850 | 2.4 | 5.7 |
| 0.1 M $Cl^-$ | 2.9 | 750 | 885 | 2.5 | 5.7 |
| 0.1 M Tris–0.01 M $CaCl_2$[a] | 7.1 | 740 | 890 | 2.8 | 5.2 |
| 8 M urea–0.01 M $Cl^-$ | 4.0 | 1330 | 1820 | 3.4 | 9.4 |
| 8 M urea–0.1 M $Cl^-$–0.018 M MEt[b] | 4.0 | 1440 | 1940 | 4.0 | 10.0 |
| | | Whole Trypsin | | | |
| 0.1 M $Cl^-$–0.01 M phosphate | 6.8 | 800 | 940 | 2.4 | 6.1 |
| 0.1 M $Cl^-$ | 3.0 | 730 | 880 | 2.3 | 6.0 |
| 8 M urea–0.1 M $Cl^-$ | 4.0 | 1200 | 1660 | 3.1 | 9.6 |
| 8 M urea–0.1 M $Cl^-$–0.018 M MEt[b] | 4.0 | 1400 | 1800 | 3.7 | 10.0 |
| | | $\alpha$-Trypsin | | | |
| 0.1 M Tris–0.01 M $CaCl_2$ | 7.1 | 805 | 770 | 2.7 | 4.2 |
| 0.1 M $Cl^-$–0.01 M $CaCl_2$ | 3.0 | 810 | 770 | 2.7 | 4.2 |
| | | $\beta$-Trypsin | | | |
| 0.1 M Tris–0.01 M $CaCl_2$ | 7.1 | 860 | 895 | 2.7 | 5.2 |
| 0.1 M $Cl^-$–0.01 M $CaCl_2$ | 3.1 | 840 | 900 | 2.6 | 5.2 |

[a] Trypsinogen purified by SE-Sephadex (C-50) chromatography (see Methods). The rest of the trypsinogen data refers to unfractionated commercial material. [b] MEt., 2-mercaptoethanol. The proteins referred to were reduced in 8 M urea at pH 8.5 by use of 2-mercaptoethanol as described by Herskovits and Laskowski (1961a), followed by acidification.

results obtained on a single protein (Worthington), isolated and purified by the same procedures as those used for the isolation of the three trypsin components (Schroeder and Shaw, 1968). Differences in the $\Delta\epsilon_M$ values of the same fractions of the enzyme and zymogen, obtained with a given perturbant were found to be within the experimental uncertainties of the difference spectral method (±5 to ±7%). The last two columns of this table and the following Table IV list estimates of the average number of exposed tryptophyl and tyrosyl residues, based on the best fits of the data. The sucrose values given in parentheses in the last column were not included in the final averages because of their high values. The high $\Delta\epsilon_M$ values at 284–286 mμ and the resultant high estimates of tyrosyl exposure obtained with this perturbant may be attributed to specific binding of sucrose to certain tyrosine-containing areas of the enzyme or, perhaps, due to the effects of association or dimerization (Cunningham *et al.*, 1953) induced by the presence of sucrose. Similar effects have been noted in cases of several other proteins (Herskovits and Laskowski, 1962b; Kronman and Holmes, 1965; Herskovits and Sorensen, 1968b) which makes this perturbant less than ideal for routine perturbation studies. It should be noted, however, that sucrose had no adverse effects on the tyrosyl perturbation spectra of trypsinogen (Table III).

Chromatography had little or no effect on the perturbation parameters of trypsinogen (Table IV). The data obtained with the commercial protein before chromatography gave average exposures ranging from 1.5 to 3.4 tryptophyls and 4.6 to 7.5 tyrosyls, with an average values of 2.6 ± 1 tryptophyls and 6.3 ± 1 tyrosyl groups exposed. These estimates are based on the average results obtained with six perturbants, 20% dimethyl sulfoxide, glycol, glycerol, propylene glycol, tetramethylurea, and hexaethylene glycol. After chromatography, the average exposures based on the data of eight perturbants listed in Table III were 2.5 ± 1 tryptophyl residues and 6.6 ± 1 tyrosyl residues exposed. The average exposures obtained with unfractionated trypsin were 1.3 to 3.4 tryptophyls and 6.5 to 8.0 tyrosyls, using the same six perturbants as for trypsinogen, with average values of 2.6 ± 1 tryptophyls and 7.3 ± 1 tyrosyls exposed. On the average the exposure of tryptophyls is only slightly higher than in the active, $\alpha$- and $\beta$-trypsins. On the other hand, about two more tyrosyl groups seem to be exposed in the unfractionated parent protein than in the two active fractions (Table III). This is undoubtedly due to the difference spectral contribution of the nearly fully exposed tyrosyls in the inert fraction of trypsin,[1] comparable to the exposure in the urea-denatured enzyme (Tables III and IV).

Lowering the pH of the enzyme and zymogen solutions had no significant effect on the tyrosyl and tryptophyl exposure (Figure 2 and Table IV) comparable to the effect of pH on the reactivity of the latter groups with *N*-bromosuccinimide (Spande *et al.*, 1966).

*Optical Rotatory Dispersion and Circular Dichroism Measurements.* Subtle differences in the solution conformation of

[1] With about 30% inert material and 30 and 40% $\alpha$- and $\beta$-trypsin present in the average commercial trypsin preparation (Schroeder and Shaw, 1970; Sipos and Merkel, 1970; and Table I), the average estimate of tryptophyl and tyrosyl exposure based on the data of the three chromatographed enzyme fractions (Table III) is 2.8 tryptophyl and 6.8 tyrosyl groups exposed, relatively close to what is actually found.

$\alpha$- and $\beta$-trypsin are also characterized by differences in the optical rotatory dispersion (ORD) and circular dichroism (CD) spectra in the peptide-absorbing ultraviolet region (Figure 4). Significant differences are noted in both the ORD and CD spectra of the inert trypsin component (data represented by dotted lines in Figure 4). The relatively low amplitude in the CD spectrum of this component at 207 and 220 m$\mu$, suggests the loss of some $\alpha$-helical structure and possibly also some $\beta$ structure upon inactivation or autolysis of the active enzyme components.

The ORD spectra of $\beta$-trypsin and the unfractionated parent material have been examined in connection with possible effects of some of the 20% perturbants used for our difference spectral measurements. Measurements on aqueous solutions in the presence and absence of 20% ethylene glycol, glycerol, propylene glycol, and hexaethylene glycol indicate no significant changes in the ORD spectra in the neighborhood of 230 m$\mu$, suggesting that these perturbants have no adverse effects on the solution conformation of trypsin. The mean residue rotation, $[m']_\lambda$, at 230 m$\mu$ obtained on $\beta$-trypsin solutions at pH 7.1 with and without these perturbants was $-1860 \pm 50°$. Similar results were obtained with whole trypsin.

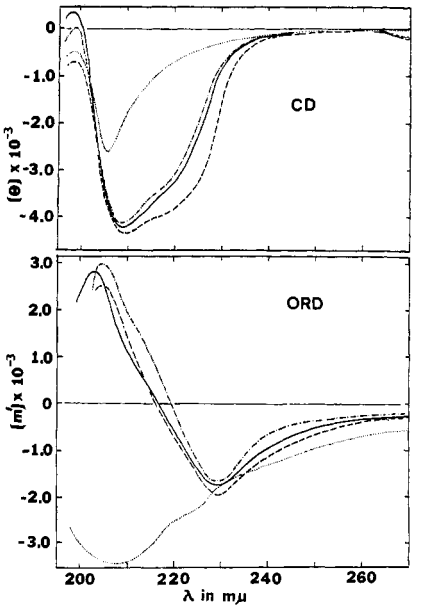


FIGURE 4: The circular dichroism (CD) and optical rotatory dispersion (ORD) spectra of $\alpha$-trypsin (-·-·-·-), $\beta$-trypsin (———), inert trypsin (.....), and trypsinogen (- - -) in 0.1 M, pH 7.1, Tris buffer–0.01 M $CaCl_2$. The protein concentrations used ranged from 0.018 to 0.036%. Cylindrical cells of 0.10- and 1.0-cm path length were used.

## Discussion

The solvent perturbation data obtained on the two enzymatically active trypsin components $\alpha$- and $\beta$-trypsin (Schroeder and Shaw, 1968) indicate that on an average about four to six of the ten tyrosyl residues and two to three of the four tryptophyl residues are exposed and accessible to solvent (Table III). These results are generally consistent with the observations that five or six of the tyrosyls seem to be reactive toward tetranitromethane (Kenner *et al.*, 1968) and cyanuric fluoride (Hachimori *et al.*, 1966) and are found to ionize rapidly and reversibly (Smillie and Kay, 1961; Inada *et al.*, 1964; Lazdunski and Delaage, 1965). As in the case of the solvent perturbation of the unfractionated parent material (Table IV and Herskovits and Villanueva, 1969), the higher value of 6.7 reactive groups obtained with *N*-acetylimidazole (Riordan *et al.*, 1965) probably represents the additional uptake of reagent by the nearly fully exposed tyrosyls of the inert component of trypsin (Table III).

In comparison with $\alpha$- and $\beta$-trypsin, the data obtained on trypsinogen indicates that on an average about one to 1.6 additional tyrosyl residues are exposed in the precursor of the enzyme (Table III). There is little or no change in the exposure of the tryptophyl residues. Changes in the environment of some of the tyrosyl residues in the activation process have been suggested, based on difference spectral changes (Delaage and Lazdunski, 1965; Benmouyal and Trowbridge, 1966) and recently, by the changes in fluorescence quantum efficiency of a dansyltyrosyl derivative of trypsinogen, following activation (Kenner and Neurath, 1971). Kenner and Neurath (1971) have suggested that the observed changes in the fluorescence properties of this derivative of trypsinogen during the course of activation are due to a rearrangement of the peptide chain around the cluster of tyrosyl residues 11, 28, and 137, and the fact that in the activated enzyme the new amino-terminal end of the peptide chain is closely situated in the fluorophore, dansyltyrosine 137. The reactivity of the tyrosyl residues remains unaltered toward tetranitromethane during the course of activation (Kenner *et al.*, 1968). Since the optical rotatory dispersion and circular dichroism spectra of the enzyme and zymogen are also largely unaltered in the peptide-absorbing far-ultraviolet region (Figure 4), it appears that the activation of trypsinogen must be accompanied by relatively subtle changes in the active site and the neighboring surface regions of the protein.[2]

Commercial trypsin preparations appear to be more reactive toward both cyanuric fluoride (Hachimori *et al.*, 1966; Gorbunoff, 1969) and *N*-acetylimidazole (Riordan *et al.*, 1965; Gorbunoff, 1969) than trypsinogen preparations. Activation of trypsinogen is accompanied by an apparent increase in reactivity from a maximum of five to six tyrosyl residues with the former and from five to nearly seven residues with the latter reagent. In the enzyme one to two tyrosyls appear to be normalized (Smillie and Kay, 1961; Inada *et al.*, 1964; Lazdunski and Delaage, 1965). The comparison of the solvent perturbation data obtained on whole trypsin (Herskovits and Villanueva, 1969) and the two active $\alpha$ and $\beta$ fractions of the enzyme (see Results and Table III) clearly show that of the order of two additional tyrosyl groups are exposed in the average commercial trypsin preparation. Chromatography of such trypsin preparations on SE-Sephadex (C-50) columns (Schroeder and Shaw, 1968; Sipos and Merkel, 1970) clearly shows that the activation of trypsinogen results in the generation of a fairly sizable fraction of inert trypsin. Thus while the average trypsinogen preparation seems to contain relatively little inert protein and active $\alpha$- and $\beta$-trypsin, as much as 20–30% of the average trypsin preparation consists of inert material, that, by criteria of difference spectroscopy (Tables III and IV) and optical rotation (Figure 4), appears to be extensively if not fully unfolded. In view of these observations a critical reexamination of titration behavior and chemical re-

[2] It is significant that the activation of both trypsinogen (Neurath *et al.*, 1956; Pechère and Neurath, 1957) and chymotrypsinogen (Neurath *et al.*, 1956) are accompanied by an increase in specific rotation at the sodium D line. Despite this the ORD and CD spectra in the ultraviolet region (Figure 4) indicate little or no change in helix content upon activation of trypsinogen.

activity of the purified trypsin fractions toward the latter reagents should be of great interest.

The difference spectra and optical rotatory dispersion of the two active trypsin fractions reveal only relatively minor differences (Table III and Figure 4). The $\alpha$-enzyme which has an interchain split between residues 131 and 132 (Schroeder and Shaw, 1968), not very far removed from tryptophyl residue 127, seems to have a slightly tighter conformation. The apparent burial of about 0.5 tyrosyl and 0.2 tryptophyl seeems to alter slightly both the amidase specificity (Schroeder and Shaw, 1968) and the efficiency of quenching of tryptophyl fluorescence in dansyltyrosyl trypsin (Kenner and Neurath, 1971). Sipos and Merkel (1970) have found that $\alpha$-trypsin in the presence of a calcium ion can be readily converted into a more compact and more active calcium-stabilized form of trypsin characterized by the development of a slightly positive difference spectrum at 20–40° (with trypsin in 0.5 M NaCl at 20° used as a reference). The $\beta$ enzyme appears to have a somewhat less compact conformation and appears to be less readily converted to the calcium-stabilized, more active form (Sipos and Merkel, 1970).

A comparison of the difference spectral data of the two active forms of trypsin and trypsinogen given in Table III shows that 1.6 to 3.4 tryptophyl residues are "exposed" to the various perturbants used in this study. Despite the fact that there are nearly twice as many tryptophyl residues in chymotrypsin, the exposure of tryptophyls based on solvent perturbation is nearly the same in both trypsin and chymotrypsin. In the latter enzyme 2.1 to 3.7 groups based on a total of seven are exposed to the same perturbants (Williams *et al.*, 1965). The perturbation of the tryptophyl residues in the two enzymes thus supports the notion (Kenner and Neurath, 1971) that the enzymes have closely similar three-dimensional structures in solution, dictated by the homology of their primary sequence (Walsh and Neurath, 1964). Unfortunately, there is relatively little information concerning the chemical reactivity of the tryptophyl residues in these two enzymes that may be compared with the solvent perturbation data.

In agreement with our findings, two of the four tryptophyl residues in trypsin as well as trypsinogen are readily oxidized by hydrogen peroxide, while a third group is found to react more slowly at higher concentrations (Hachimori *et al.*, 1964). Unfortunately, the reactivity with *N*-bromosuccinimide (Spande *et al.*, 1966) and the Koshland reagent, 2-hydroxy-5-nitrobenzyl bromide (Delaage *et al.*, 1968) suggests a more complicated and unsettled picture. One-half to 2.7 groups are oxidized in the pH 7.0 to pH 5.0 range with the former reagent in both trypsin and trypsinogen, and only 0.1 of a group with the latter, in the case of trypsinogen at pH 7.0. In the more acidic region between pH 4.5 and 3.5, from 3.2 to 3.8 groups react with *N*-bromosuccinimide, despite the fact that neither the optical rotation (Neurath *et al.*, 1956) nor the solvent perturbation difference spectra (Figure 2 and Table IV) reveals any pronounced changes in protein conformation as a function of pH.[3] The solvent perturbation and reactivity data with hydrogen peroxide (Hachimori *et al.*, 1964) suggest that in both trypsin and trypsinogen two or perhaps three groups should occupy surface positions that are fairly accessible to reagent and solvent. On the other hand, the reactivity with the Koshland reagent[4] and *N*-bromosuccinimide would indicate that several if not all the tryptophyls must be at least partly blocked to reagent access.

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[3] The problems attendant upon the interpretation of reactivity data with *N*-bromosuccinimide have been discussed by Kronman *et al.* (1967) and Kronman and Robbins (1970).

[4] The reactivity of the tryptophyl residues in chymotrypsinogen A and B with this reagent is also found to be too low. Only about 0.3 of a tryptophan is found to react at pH 7.7 (Delaage *et al.*, 1968). Similarly in lysozyme, only 0.5–1.5 groups are found to react (Dasgupta *et al.*, 1965; Bewley and Li, 1965). On the other hand, five of the six groups in lysozyme are readily oxidized by hydrogen peroxide, with the sixth group reacting at higher reagent concentration (Hachimori *et al.*, 1964). Reaction of the tryptophyls with the latter reagent are somewhat on the high side, but closer to what one would expect based on the X-ray crystallographic and solvent perturbation data (Blake *et al.*, 1967; Williams *et al.*, 1965). Interestingly enough, the relatively high perturbations of the tryptophyls in lysozyme (Williams *et al.*, 1965; Bello, 1970) are comparable to the values found for trypsin and trypsinogen.

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# Cytidine Triphosphate Synthetase. Covalent Intermediates and Mechanisms of Action*

Alexander Levitzki† and D. E. Koshland, Jr.‡

ABSTRACT: The mechanism of CTP-synthetase from *Escherichia coli* B was elucidated and appears to be composed of the steps given in the adjacent column.

Ammonia can replace glutamine as the amino donor. Evidence is presented that externally added ammonia and the nascent ammonia released from the glutamine reaction occupy the same site.

$$\text{enzyme} + \text{glutamine} \xrightarrow{\text{GTP}} \text{glutamyl-enzyme} + NH_3 \text{ (bound)}$$

$$\text{glutamyl-enzyme} + H_2O \longrightarrow \text{enzyme} + \text{glutamate}$$

$$NH_3 + \text{UTP} \rightleftharpoons H_2N\text{–UTP (tetrahedral adduct)}$$

$$H_2N\text{–UTP} + \text{ATP} \rightleftharpoons H_2N(\text{P})\text{>UTP (phosphorylated tetrahedral adduct)}$$

$$H_2N(\text{P})\text{>UTP} \longrightarrow \text{CTP} + P_i$$

CTPS[1] was first described by Lieberman (1955, 1956) and was shown to catalyze the formation of CTP from $NH_3$ and ATP (eq 1). Chakraborty and Hurlbert (1961) and Long and

$$\text{UTP} + NH_3 + \text{ATP} \xrightarrow{Mg^{2+}} \text{CTP} + \text{ADP} + P_i \qquad (1)$$

Pardee (1967) established that the enzyme can also utilize glutamine as a nitrogen donor when GTP is present as an allosteric effector.

$$\text{UTP} + \text{Glu-NH}_2 + \text{ATP} \xrightarrow[\text{GTP}]{Mg^{2+}} \text{CTP} + \text{Glu·OH} + \text{ADP} + P_i \qquad (2)$$

The ever increasing literature on allosteric proteins is revealing more and more of their behavior. On the other hand the complete explanation of the binding and catalytic behavior of a protein in terms of its structure is yet to be performed for any one enzyme. CTP-synthetase seemed to offer a particularly advantageous case for such a study. Its structure is relatively simple since its subunits are identical (Levitzki *et al.*, 1971), and its association–dissociation behavior from a tetramer to dimer also is conveniently simple (Long *et al.*, 1970). Its interaction with ligands showed both negative (Levitzki and Koshland, 1970) and positive cooperativity (Long and Pardee, 1967; Levitzki and Koshland, 1969) and it is one of that fairly large group of enzymes which can use either glutamine or ammonia as a nitrogen source. It thus appeared sufficiently complicated to exhibit properties which are illustrative for many allosteric proteins and yet sufficiently simple to allow the hope that its properties could be described in terms of its molecular structure. In this paper the covalent chemistry of the enzyme and the relation between the ammonia and glutamine sites are elucidated. In subsequent papers the allosteric and structural properties will be examined.

## Experimental Section

The nucleotides and other chemicals were obtained commercially and were of the highest purity available. In cases in which precise stoichiometric determinations were needed, chromatographic tests were performed to establish the purity of the reagents. $\beta,\gamma$-NH-ATP (referred to as ADPNP) was the gift of Dr. Ralph Yount of Washington State University, Pullman, Wash. [$^{14}$C]Glutamine (New England Nuclear or

* From the Department of Biochemistry, University of California, Berkeley, California. *Received April 13, 1971.* This work was supported in part by research grants from the National Science Foundation (GB-7057) and the U. S. Public Health Services (AM-GM-09765).

† Present address: Department of Biophysics, The Weizmann Institute of Science, Rehovot, Israel.

[1] Abbreviations used are: cytidine 5′-triphosphate synthetase, CTPS; tetrahedral adduct of $NH_3$ to 4′ position of UTP, $NH_2$-UTP; intermediate in which OH at 4′ position of $NH_2$-UTP is phosphorylated, $NH_2(\text{P})$>—UTP; $\beta,\gamma$-imido-ATP, ADPNP; polyethyleneimine, PEI; 6-diazo-5-oxonorleucine, DON; *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, HEPES.