Ligand-Induced pK Changes in Chymotrypsin*

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ABSTRACT: When neutral ligands, such as anisole and coumarin, are added to an unbuffered α-chymotrypsin solution in the pH range 6–7.5, the release of a proton can be detected. In the pH range 7.5–9 the uptake of a proton is seen. The magnitude of this effect is related to ligand concentration in a way that indicates complexing with the enzyme at the substrate binding site. These data show that binding of a ligand is associated with the lowering of the pK of a group originally with pK = 6.7 and the raising of the pK of a group originally with pK = 8.6. Formanilide shows an effect only on the pK = 8.6 group. Several other ligands, including L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone, were tested. These experiments are interpreted as indicating that the ammonium–carboxylate salt bridge and the imidazole–hydroxyl hydrogen bond shown to exist in the tosylchymotrypsin crystal are formed or strengthened by the complexing of a ligand.

Chemical evidence has indicated the existence of a hydrogen-bonded serine–histidine pair at the catalytic site of chymotrypsin and an N-terminal isoleucine-α-ammonium–carboxylate salt bridge necessary to maintain the integrity of the enzyme's binding site (Bernhard, 1968, p 238ff). The recent X-ray crystallographic studies of tosylchymotrypsin have confirmed these inferences (Mathews *et al.*, 1967), but still to be settled is whether these intramolecular interactions are present in the native enzyme which is not associated with a ligand. This is an important issue because the tosyl group has a substrate-like structure; indeed, tosyl chloride is an efficient and selective chymotrypsin active site reagent because of this similarity. The binding of a ligand to chymotrypsin, as indicated by the K_m of ATA,[1] depends upon a dissociating group with a pK near 8.3 (Himoe and Hess, 1966). Because this pK is close to that for a transition in the enzyme detected by a change in specific rotation and associated with the salt bridge involving the α-ammonium group of isoleucine 16, the binding site of the enzyme is believed to be associated with this salt bridge (Oppenheimer *et al.*, 1966). If the dissociating group can affect binding of ligands, then the binding of ligands would be expected to affect the dissociating group. Previous experiments have not been designed to examine small changes in pK values. The studies presented here deal with small changes in pK values which occur when chymotrypsin binds ligands.

Materials

Three-times-crystallized α-chymotrypsin, three-times-crystallized chymotrypsinogen A, and salt-free two-times-crystallized trypsin were purchased from Worthington Biochemical Corp.; ATEE from Mann Research Laboratories; TPCK from Cyclo Chemical Co.; anisole from The Matheson Co., Inc.; benzenesulfonamide and formanilide from Distillation Products Industries; coumarin and α-naphthol from Aldrich Chemical Co., Inc.; DFP from Boots Pure Drug Co.; Tris from Sigma Chemical Co.; and standard NaOH solutions from Anachimia Chemicals, Ltd.

A sample of purified chymotrypsin was prepared by passing a solution of commercial chymotrypsin over a Sephadex G-25 column equilibrated with 0.001 M HCl.

Chymotrypsinogen free of chymotrypsin was prepared by treating commercial chymotrypsinogen at 4° with 0.001 M DFP at pH 7.2 in 0.05 M sodium phosphate buffer for 4 hr, followed by dialysis against 0.001 M HCl, and lyophilization. Because this treatment did not completely abolish chymotryptic activity, the procedure was repeated with 0.004 M DFP. The preparation then had no chymotryptic activity, but incubation with 4% by weight trypsin within 4 hr released chymotryptic activity corresponding to 43% of the DFP-treated material being chymotrypsinogen.

DL-TPMK was synthesized from DL-3-amino-4-phenyl-2-butanone hydrochloride (purchased from Cyclo Chemical Co.) by reaction with tosyl chloride in pyridine overnight at room temperature. The product was recrystallized from acetone and petroleum ether to a melting point of 103.5–104.5° uncor. Elemental analysis (Schwartzkopf Microanalytical Laboratory) showed the following. *Anal.* Calcd for $C_{17}H_{19}NO_3S$: C, 64.33; H, 6.03. Found: C, 64.39; H, 5.96.

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[1] Abbreviations used: ATA, *N*-acetyl-L-tryptophanamide; ATEE, *N*-acetyl-L-tyrosine ethyl ester; DIP-chymotrypsin, diisopropylphosphochymotrypsin; TPCK, L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone; TPMK, (1-tosylamido-2-phenyl)ethyl methyl ketone.

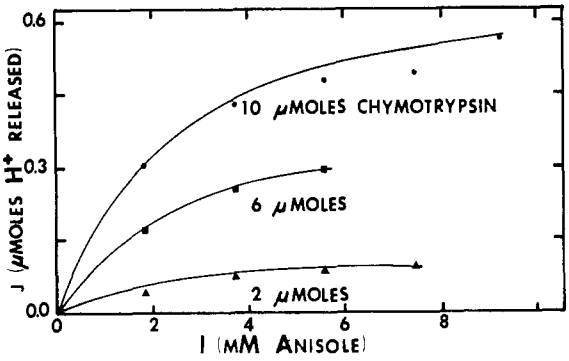

FIGURE 1: The ligand-dependent release of protons to the medium at three levels of chymotrypsin. Conditions: 0.10 M KCl, pH 6.5, and 30°.

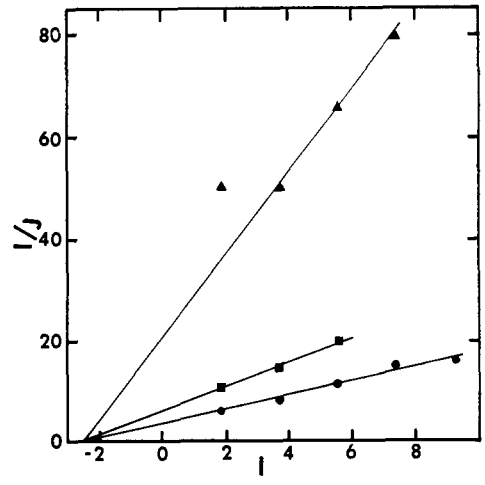

FIGURE 2: Graphical determination of K_i and J_{max} for anisole at pH 6.5. The data from Figure 1 are plotted to give as the intercept on the abscissa, $-K_i$, and as the slope, $1/J_{max}$. The triangles are for 2 μmoles, the squares for 6 μmoles, and the circles for 10 μmoles of chymotrypsin.

Methods

Assays for chymotryptic activity were performed using a Radiometer TTT1 titrator and ABU1 autoburet unit, adapted to a Leeds and Northrup Speedomax G recorder. The flexible cable from the autoburet unit drove a ten-turn helipot to give, through a balanced bridge circuit, an output equivalent to one, four, or ten times the full-scale deflection of the recorder. Enzyme activity was determined from the rate at which 0.050 M NaOH was used to maintain a given pH (7.8, unless otherwise noted) when chymotrypsin (usually 1–5 μg dissolved in 50 μl of water) hydrolyzed ATEE (added in 50 μl of acetonitrile) in 4.00 ml of water containing 0.02 mmole of Tris, 0.4 mmole of KCl, and 0.08 mmole of $CaCl_2$ at 30°. When an inhibitor was present in the assay, it was added in 50 μl of methanol, and the same volume of methanol was added to each assay in the corresponding control series.

Chymotrypsin was inactivated by TPCK at pH 7.0 at 30° in a 10-ml solution containing 10 mg of chymotrypsin, 1 mmole of Tris, and 0.2 mmole of $CaCl_2$ (or 1 mmole of sodium phosphate and no $CaCl_2$), and a varying amount of TPCK in 0.10 ml of isopropyl alcohol. At five timed intervals between 0.5 and 10–30 min, depending upon the TPCK concentration, 100-μl aliquots were removed and added to 2.00 ml of a pH 4.0 solution containing 0.1 mmole of sodium acetate. Aliquots of these diluted samples were then assayed for enzyme activity.

The short-term rate of inactivation of chymotrypsin by TPCK at 30° was determined at pH 6.5 with 49.5 ml of a solution containing 250 mg of chymotrypsin, 5 mmoles of KCl, and 1 mmole of $CaCl_2$. Before addition of inhibitor, two 100-μl aliquots were taken and added to 5.00 ml of 0.01 M HCl at 0°. Then 0.500 ml of 0.035 M TPCK in methanol was added to the enzyme. At 30 and at 111 sec 100-μl aliquots were removed and diluted as were the two before. The four diluted aliquots were then assayed for enzyme activity.

The release of protons from chymotrypsin was measured in the titrator–buret–recorder apparatus described above. At 30° a solution containing 250 mg (except as noted) of chymotrypsin, 5 mmoles of KCl, and 1 mmole of $CaCl_2$ was adjusted to the desired pH and to 49.75 ml. The compound being investigated was dissolved in methanol and a 100-μl portion was added to the chymotrypsin solution. The volume of 0.050 M NaOH required to regain the original pH was recorded. The procedure was then repeated until a total of five portions had been added. At the higher pH range (8–9), autodigestion and some absorption of atmospheric CO_2 caused a high enough background rate of alkali addition that the uptake of protons on addition of a ligand was seen as a temporary cessation of the background addition of alkali. The linear rate of renewed alkali addition was extrapolated to that time when the ligand was added and the magnitude of the proton uptake was then calculated. Because of this complication the data for the higher pH range are not so accurate as those for the lower pH range.

When the ligand tested was TPCK, the experiment was performed as above, but only one addition of TPCK was used to elicit proton release or uptake in a single experiment. The same experiment at pH 6.5 was performed on trypsin and on chymotrypsin-free chymotrypsinogen.

Between experiments the electrode and reaction vessel were washed with acetone to remove traces of ligands and with 0.01 M HCl to remove traces of adsorbed protein.

Results

When anisole was added to chymotyrpsin at pH 6.5, protons were released to the medium. As the protons could not possibly have come from the anisole, they must have come from the enzyme. Figure 1 shows that the magnitude of the proton release, j, is directly proportional to the amount of enzyme present, and that there is a saturation of the binding site as the ligand concentration, I, is raised. These data from equilibrium states may be treated like kinetic data (Dixon and Webb,

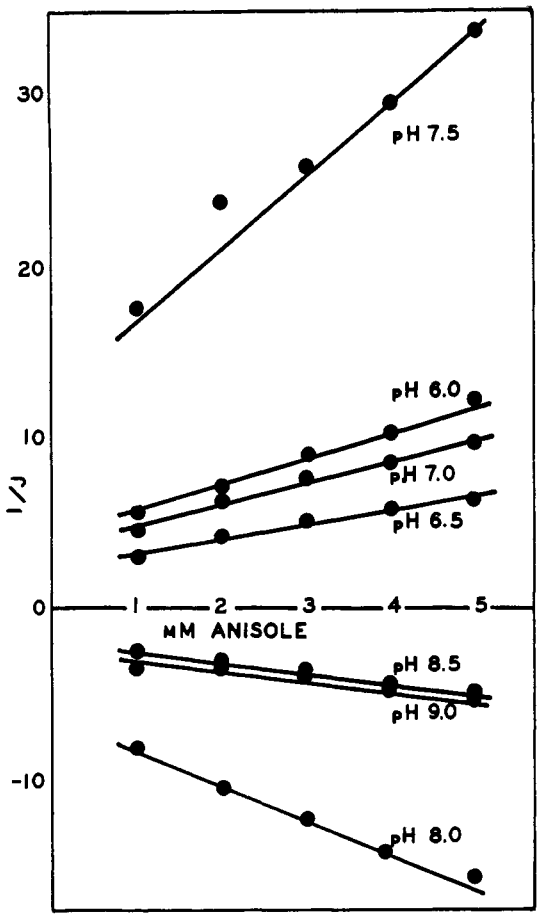

FIGURE 3: Graphical determination of J_{max} at several pH values. The anisole concentration, I, per μmole of protons released, j, is shown as a function of I for various pH values. I_{max} is the reciprocal of the slope of each line divided by the amount of enzyme present. Conditions: 0.04 mM chymotrypsin, 0.10 M KCl, and 0.02 M $CaCl_2$ at 30°.

1958, p 21) to give a binding constant, K_i.[2] In Figure 2 $1/j$ is plotted against I to give as the intercept on the abscissa, $-K_i$, and as the slope, $1/J_{max}$. K_i determined in this way is the same as the inhibition constant determined from inhibition of chymotryptic hydrolysis of ATEE. The proton-release K_i is 2.5 mM and the kinetic K_i is 2.7 mM, indicating that the release of protons occurs when anisole binds to the substrate binding site. The reciprocal of each slope in Figure 2 may be divided by the amount of chymotrypsin present to give J_{max}. This value is constant for these three experiments: 0.067 proton/anisole bound.

The proton-release experiment was performed over the pH range 6.0–9.0 and the results are shown in Figure 3. Binding of substrates becomes less tight as the pH is raised above 7.5 (Himoe and Hess, 1966) and it might be expected that binding of anisole would

[2] The protons released, j, is related to the ligand concentration, I, the dissociation constant of the enzyme–ligand complex, K_i, and the limiting value for j as I increases, J_{max}, by the equation, $j = J_{max}I/(K_i + I)$. J_{max} is always given as moles of protons released per mole of enzyme present, so that it may be taken to mean protons released per ligand molecules bound.

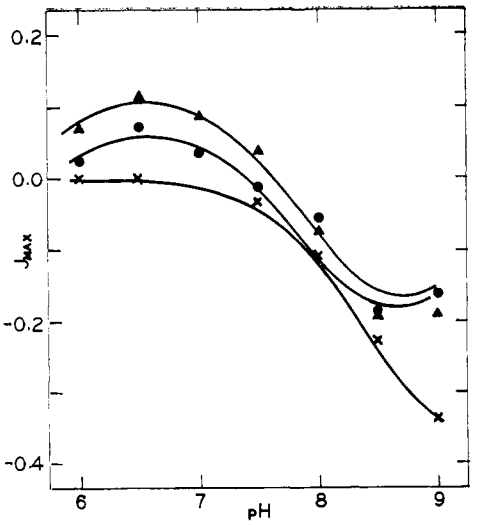

FIGURE 4: J_{max} (the protons released per ligand bound) as a function of pH. The circles are for anisole; the triangles for coumarin; and the crosses for formanilide. The lines are calculated from the pK and pK' values in Table I. Conditions: 0.04 mM chymotrypsin, 0.10 M KCl, 0.02 M $CaCl_2$, 1–5 mM anisole, 0.3–1.5 mM coumarin, and 1–10 mM formanilide at 30°.

be similarly affected. This is seen in Figure 3 by the intercepts on the abscissa occurring at $-K_i$ for pH values up to 7.5, but above that pH the intercepts are increasingly negative. When the J_{max} values, the reciprocals of the slopes, from Figure 3 are plotted as a function of pH, a complex curve is obtained (Figure 4).

The release of a proton at constant pH is due to a change *a priori* in pK of a dissociating group. The difference between the two dissociation curves characterized by pK (the original value) and pK' (the ligand induced value) is a bell-shaped proton release *vs.* pH curve. The height of this curve at any pH is J_{max}, which has been experimentally determined. The height of this difference curve at its maximum and the value of the pH at which this maximum occurs can be used to calculate pK and pK'.[3]

The same experiments were performed on other inhibitors. The results are given for coumarin and formanilide in Figure 4. The former case resembles the experiments with anisole, but the latter case shows no proton release near neutrality, only a proton uptake in the higher pH range.[4] With both these compounds the K_i values determined from proton-release data (0.8 and 3.8 mM, respectively) agreed with the kinetic K_i values.

[3] In general, when b is the fraction of the dissociating group in the unprotonated form and b' is the fraction of the perturbed dissociating group in the unprotonated form, pH = pK + log (b/(1 − b)) and pH = pK' + log (b'/(1 − b')) and their sum is 2pH = pK + pK' + log (b/(1 − b)) + log (b'/(1 − b')). At that pH where J is largest, pH = (pK + pK')/2. Therefore, at that pH, (1 − b)/b = b'/(1 − b'). As J is defined as $b' - b$, b = (1 − J)/2 and b' = (1 + J)/2 and pK = pH + log (1 + J)/(1 − J) and pK' = pH + log (1 − J)/(1 + J).

[4] It may be noted, however, that formanilide first added to chymotrypsin interfered with the release of protons subsequently elicited by anisole at pH 7.

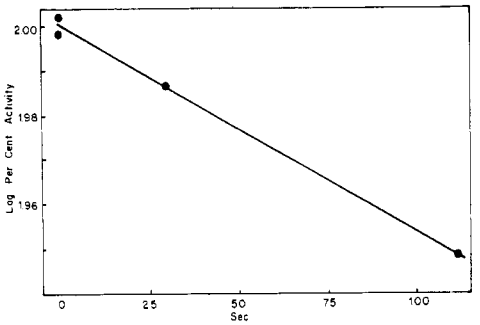

FIGURE 5: Rate of short-term inactivation of chymotrypsin by TPCK. Conditions: 0.04 mM chymotrypsin, 0.10 M KCl, 0.02 M $CaCl_2$, and 0.35 mM TPCK, at pH 6.5, 30°.

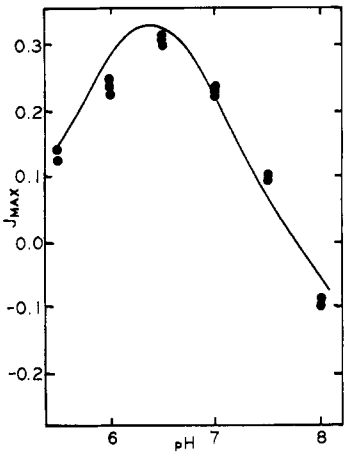

FIGURE 6: J_{max} (the protons released per TPCK bound) as a function of pH. The line is calculated from the pK and pK' values in Table I. Conditions: 0.04 mM chymotrypsin, 0.10 M KCl, 0.02 M $CaCl_2$, and 0.3 mM TPCK at 30°.

α-Naphthol, with a kinetic and proton-release K_i of 2.6 mM also elicited a proton release at pH 6.5, but, because of the acidity of the phenolic hydroxyl group, it was not investigated further. Similarly, benzenesulfonamide, with a kinetic K_i of 5.9 mM, showed a J_{max} at pH 6.5 of 0.155, but the acidity of the sulfonamide group prevented a more extensive investigation.

It was desired to test TPCK (Schoellman and Shaw, 1963) in the same manner as anisole, coumarin, and formanilide. As the proton release (or uptake) was determined at only one concentration of TPCK at a given pH, it was necessary to know the K_m[5] for this compound. The pseudo-first-order rate constant for the inactivation of the enzyme by TPCK was determined as a function of TPCK concentration. The insolubility of TPCK precluded measurements above 0.35 mM. (Substitution of phosphate buffer for Tris and $CaCl_2$ made no difference in the results.) The K_m determined by conventional graphical means was 0.3 mM. Because in these experiments TPCK was not in large excess over chymotrypsin, this estimated K_m was used as a dissociation constant to calculate the free TPCK concentration initially present in the chymotrypsin–TPCK solution. Incorporating this small correction (less than 10%), into a recalculation of K_m produced a corrected value of 0.27 mM. To confirm that the release of protons was associated with formation of a reversible enzyme–inhibitor complex and not with the following alkylation step in which HCl would be formed, the activity of chymotrypsin was determined before and immediately after addition of TPCK. The results, shown in Figure 5, indicate that in the few seconds following TPCK addition there was no sudden permanent loss of activity. During this time there was a release of protons which, if due to alkylation, would correspond to about 30% inactivation. The data shown in Figure 6, then, reflect changes in pK of groups on the enzyme. Because of the acidity of the sulfonamide hydrogen of TPCK (Kézdy *et al.*, 1967), it was not possible to extend the data to a higher pH, although it is evident that a group with pK above 8 is being affected by TPCK binding.[6] Neither trypsin nor chymotrypsinogen gave any release of protons at pH 6.5 on addition of TPCK. Because the chymotrypsin-free chymotrypsinogen contained DIP-chymotrypsin, it was also seen that this DFP-inactivated material also showed no proton release.

To assure that proton release was due to chymotrypsin itself and not any low molecular weight contaminant in the commercial preparation, a sample of chymotrypsin purified by gel filtration was tested with α-naphthol and with TPCK, giving results indistinguishable from those obtained with the commercial sample.

The pK and pK' values are presented in Table I. No pK' with TPCK for the pK = 8.6 group is shown only because it was not quantitatively evaluated, not because there was no shift of pK.

Discussion

Bender described the uptake of a proton by chymotrypsin when it binds benzyl alcohol above pH 7.5 (Bender and Wedler, 1967), but his data show, not a bell-shaped curve expected for a shift in pK, but a sigmoid curve that mysteriously indicates the complete abolition of an acidic group with pK = 8.8. A possible although not completely satisfying explanation is that there is a pK shift of more than 2 pH units, and, had it been possible to continue the experiment above pH 9.5, a bell-shaped curve would, in fact, have been found.

The magnitudes of the pK shifts reported here (Table

[5] K_m here means $(k_2 + k_3)/k_1$ in the sequence

$$RCl + EzH \underset{k_2}{\overset{k_1}{\rightleftharpoons}} [RCl\text{---}EzH] \xrightarrow{k_3} EzR + HCl$$

RCl is TPCK, EzR is the irreversibly inactivated enzyme, and the intervening species is the reversibly bound enzyme–TPCK complex.

[6] For purposes of comparison with TPCK, it was of interest to perform the proton-release experiment on the nonhalogenated analog, TPMK. When DL-TPMK was synthesized and examined, no effect could be demonstrated up to a concentration of 0.7 mM. The insolubility of TPMK prevented a closer approach to the K_i, kinetically determined to be 2 mM, which rendered this particular experiment inconclusive.

I) are not obviously related to structures of the compounds, but the results unequivocally indicate that binding of a ligand to the active site of chymotrypsin can change the pK of groups on the enzyme. Any identification of the groups whose pK values are being perturbed must be tentative, but it is tempting to identify the pK = 6.7 group as the imidazole of histidine 57 which has been shown to have pK = 6.7 and is part of the active site (the evidence is reviewed in Baker, 1967, p 130ff). The α-ammonium of isoleucine 16 might be the pK = 8.6 group because of its demonstrated interrelation with binding properties (Oppenheimer *et al.*, 1966).

X-Ray crystallography of a chymotrypsin derivative in which the active site serine is tosylated shows a salt bridge between the α-ammonium group of isoleucine 16 and the β-carboxylate group of aspartic acid 194, and a hydrogen bond between the imidazole of histidine 57 and the tosylated oxygen of serine 195 (Mathews *et al.*, 1967). The special reactivity of serine 195 as a nucleophile has been ascribed to this interaction (Cunningham, 1957). If the tosylchymotrypsin crystal is a reliable guide to the intermolecular interactions of the ligand-bound molecule in solution, we can conclude that the salt bridge and the hydrogen bond exist in a chymotrypsin–anisole (or other ligand) complex in solution.

This paper compares the ligand-bound state, in which the salt bridge and hydrogen bond exist, with the unbound state. It is suggested that when chymotrypsin binds a ligand, it is histidine 57 that becomes a stronger base and isoleucine 16 that becomes a stronger acid. This is consistent with the formation of the salt bridge and hydrogen bond accompanying the binding of a ligand. The free enzyme does not possess these interactions, the ligand-bound form does.

Bringing together an ammonium and a carboxylate group in the interior of a protein molecule is energetically favorable. Deprotonation of the ammonium group disrupts this favorable arrangement. Therefore the ammonium group as part of this pair is a stronger acid than it is alone. The carboxylate group, in the same way, becomes a stronger base; proton-release studies at a lower pH range should detect this. A similar argument applied to the histidine–serine interaction indicates that in the hydrogen-bonded pair histidine is a stronger base than it is alone. Possibly the strengthening of these interactions is achieved by complexing of the ligand with one of several conformers of the free enzyme which exist in equilibrium. This one form possesses the two intermolecular interactions and the binding site for the ligand. As the ligand binds to this conformer, the equilibrium among all the conformers is displaced in its favor. A similar explanation was offered (Hummel and Witzel, 1966) to explain the pH-dependent uptake and release of protons when ribonuclease binds nucleotides.[7] According to this interpretation of the data the effect of formanilide upon only one of the dissociating groups may mean there can be a form of chymotrypsin that has a binding site and salt bridge but not the hydrogen bond.

It is, of course, not only incidental that the bound form has the histidine–serine interaction that makes serine an effective nucleophile. It is this interaction, triggered by the binding step, that is essential for the next step in catalysis, the nucleophilic attack of the β-oxygen of serine on the carbonyl carbon of a substrate.

[7] This analysis follows logically from the studies of Lumry (Parker and Lumry, 1963; Yapel *et al.*, 1966; Lumry and Kim, 1967) on the various conformers of chymotrypsin and their varying ability to bind ligands.

TABLE I: Values of pK and pK' Measured for Various Ligands.

Inhibitor	pK	pK'	pK	pK'
Anisole	6.7	6.5	8.6	8.9
Coumarin	6.65	6.55	8.5	8.8
Formanilide	*a*	*a*	8.7	9.3
TPCK	6.7	6.1	*b*	*b*

[a] No shift in pK was detected in this pH region for formanilide. [b] There was a positive shift in the pK value in this pH range for TPCK, but because of the acidity of the sulfonamide hydrogen, the magnitude of this shift was indeterminate.

Acknowledgments

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Some Physical and Chemical Studies on Two Polypeptide Components of High-Density Lipoproteins of Human Serum*

V. Shore and B. Shore

ABSTRACT: Two different polypeptides separated from the protein moiety of high-density lipoproteins of human serum were found to be similar in molecular weight but very different in amino acid composition. One of the peptides, characterized by carboxyl-terminal glutamine, contains no histidine, arginine, tryptophan, or cysteine. Its amino acid composition is: Lys_{15},-Cys_2,Asp_5,Thr_{11},Ser_{11},Glu_{26},Pro_7,Gly_6,Ala_9,Val_{10},Met_2,-Ile_2,Leu_{14},Tyr_6,Phe_7; the total number of residues is 133 and the molecular weight from amino acid composition is 14,900. Sedimentation equilibrium experiments yielded molecular weight values of 14,300 and 14,900 for the polypeptide R-Gln in urea solutions and in guanidine hydrochloride solutions, respectively. The other polypeptide, characterized by carboxyl-terminal threonine, contains no isoleucine, cystine, or cysteine. Its amino acid composition is: Lys_{10},His_3,Arg_9,-Asp_{13},Thr_5,Ser_8,Glu_{24},Pro_5,Gly_6,Ala_{10},Val_7,Met_2,Leu_{20},-Tyr_4,Phe_3,Trp_4; the total number of residues is 133 and the molecular weight from amino acid composition is 15,500.

Sedimentation equilibrium experiments on the polypeptide R-Thr in guanidine hydrochloride solutions indicated homogeneity with respect to molecular weight; however, the molecular weight value 31,400 indicates that the polypeptide R-Thr exists as a dimer in guanidine hydrochloride solutions. Sedimentation equilibrium experiments on the polypeptide R-Thr in urea solutions and in dilute salt solutions containing sodium dodecyl sulfate indicated heterogeneity with respect to molecular weight and weight-average molecular weights of approximately 20,000.

The protein moiety of high-density lipoproteins (1.065–1.195 g/cc) of human serum is heterogeneous (Shore and Shore, 1968a,b). Polypeptides with carboxyl-terminal glutamine and threonine were found in comparable amounts by hydrazinolysis and by carboxypeptidase experiments on the lipid-free proteins from the 1.126–1.195-g/cc (HDL_3) and the 1.083–1.124-g/cc (HDL_2) lipoprotein fractions.

The present report describes some physical properties, obtained principally by the method of equilibrium sedimentation, and the amino acid compositions of these two polypeptide subunits which were separated by DEAE-cellulose chromatography in 8 M urea.

Experimental Section

Materials. The lipoproteins of density 1.126–1.195 g/cc (HDL_3), which were used as the source of protein in these experiments, were isolated from human serum by ultracentrifugation as described previously (Shore and Shore, 1967). The protein moiety was obtained with essentially complete recovery in lipid-free (<0.01% P; <0.02% cholesterol), water-soluble form by extraction with mixtures of ether and ethanol as described before (Shore and Shore, 1967). Ultra Pure urea and guanidine hydrochloride from Mann Research Laboratories, New York, N. Y., were used without further purification. Triton X-100 (alkylphenoxypolyethoxy ethanol) is a product of Rohm and Haas, Philadelphia, Pa. DEAE-cellulose in a microgranular, fully swollen, and wet form was obtained from Reeve Angel and Co., Clifton, N. J. Carboxypeptidases A (COA-DFP) and B (COB-DFP) were obtained from Worthington Biochemical Corp., Freehold, N. J.

DEAE-cellulose Chromatography. The protein moiety of HDL_3 lipoproteins was chromatographed at 6° on DEAE-cellulose columns (0.9 × 27 cm) which had been equilibrated with the starting buffer, 0.005 M Tris-HCl (pH 8.0) containing 8 M urea. The ion binding capacity was 0.2 mequiv/ml; the capacity for the protein under investigation was not established although it was found to be at least twice the amount used in these experiments. A linear gradient for elution of the peptides was formed by pumping 0.125 M Tris-HCl (pH

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