Synthesis of Peptide Bonds by Proteinases. Addition of Organic Cosolvents Shifts Peptide Bond Equilibria toward Synthesis†

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ABSTRACT: We found that in 60% (v/v) glycerol the equilibrium constant for the hydrolysis of the Arg^{63}-Ile peptide bond in soybean trypsin inhibitor (Kunitz) at pH 5.00 is 0.5, while in water it is 4.0. To show that organic cosolvents generally favor peptide bond synthesis, we measured their effect upon K_{syn} of benzyloxycarbonyl-L-tryptophanylglycineamide by monitoring with high-performance liquid chromatography the chymotrypsin catalyzed hydrolysis and formation of this compound in the presence of excess glycineamide. In water K_{syn} is 0.45 M^{-1}; it rises upon addition of all organic cosolvents tried. The pH dependence of K_{syn} in water and in 60% (v/v) triethylene glycol is in accord with thermodynamic prediction. The cosolvent concentration cannot be increased indefinitely due to loss of enzyme activity and of solubility of reactants. The largest K_{syn} value obtained was 38 M^{-1} in 85% (v/v) 1,4-butanediol. This cosolvent was then employed for trypsin and chymotrypsin catalyzed addition of glycineamide and of L-methionineamide to the COOH termini of several peptides. The latter may provide a general route for attachment of peptides obtained by enzymatic hydrolysis to solid supports prior to solid phase sequencing. The large increases in K_{syn} observed upon addition of organic cosolvents occur in spite of only moderate reduction of water activity as revealed by vapor pressure measurements. We therefore surmised that the main cause of the increase in K_{syn} is the diminution in the equilibrium constant for proton transfer from the COOH to NH_2 termini of substrates. A plot of the logarithm of a new hybrid equilibrium constant, §, vs. the change in the logarithm of the proton transfer equilibrium constant shows that this surmise is semiquantitatively valid.

Protein semisyntheses could be carried out very conveniently if protein fragments obtained by the action of proteinases could be recombined again by the action of the same proteinases. In order to achieve such an objective, techniques are needed to shift the conditions in such a manner that the enzymes remain catalytically active, and under one set of conditions peptide bond hydrolysis is favored, and under the other, peptide bond synthesis is favored.

Interest in this problem was very high when the possibility was entertained that in vivo protein synthesis may be carried out by proteinases. The classic review by Borsook (1953) describes what was then known about peptide bond hydrolysis equilibria. Since it became clear that proteins are synthesized in vivo on ribosomes, the interest in studying and manipulating peptide bond hydrolysis equilibria largely waned. Among few exceptions to this neglect was the work on protein–proteinase inhibitors.

Finkenstadt & Laskowski (1965) and Ozawa & Laskowski (1966) showed that carboxypeptidase B specifically removes Arg^{63} from modified (i.e., an inhibitor whose reactive site peptide bond Arg^{63}-Ile is hydrolyzed) soybean trypsin inhibitor (Kunitz) and thus renders the inhibitor inactive. Sealock & Laskowski (1969) incubated the des-Arg^{63} modified inhibitor with large quantities of arginine, trypsin, and carboxypeptidase B and succeeded in adding the removed Arg^{63} residue back to the des-Arg^{63} inhibitor. The driving force for the reaction was the restoration of inhibitory activity and the subsequent association of the modified inhibitor with trypsin. Niekamp et al. (1969) and later, Mattis & Laskowski (1973), showed that the equilibrium constant for the hydrolysis of the reactive site Arg^{63}-Ile in soybean trypsin inhibitor is near unity at neutral pH but strongly favors hydrolysis as the pH departs significantly from neutrality. Thus hydrolysis could be carried out to near completion at acidic pH values and a subsequent pH change would allow for partial resynthesis. Wang & Laskowski (1972; see Kowalski et al., 1974) added 3 M glycineamide and trypsin to modified soybean trypsin inhibitor (Kunitz), whose NH_2 terminus was specifically blocked, and attained roughly equimolar equilibrium between modified blocked inhibitor and an . . . $Arg^{63}Gly^{63A}NH_2$ adduct of the modified and blocked inhibitor. When 3 M glycineamide was removed, trypsin hydrolyzed the ArgGly bond in the adduct essentially to completion.

These three techniques, coupling of peptide bond synthesis to the formation of protein–ligand complex, manipulation of pH, and the use of mass action, allowed us (Kowalski et al., 1974) and others (Jering & Tschesche, 1976; Odani & Ikenaka, 1978) to prepare a number of derivatives of protein proteinase inhibitors in which one specific residue was replaced by another amino acid residue. While these proved useful in increasing our understanding of enzyme–inhibitor interactions and of the evolution of inhibitor specificity, the methods employed were of rather limited generality.

We were therefore anxious to find a more general method of perturbing the peptide bond hydrolysis equilibrium toward synthesis. An experiment performed for another purpose provided a clue. We had carried out the hydrolysis and resynthesis of reactive site peptide bond in soybean trypsin inhibitor (Kunitz) in the absence of (Mattis & Laskowski, 1973) and in the presence of 60% (v/v) glycerol (Figure 1). To our surprise, in 60% glycerol K_{hyd} was a factor of 8 lower. The question now arose whether the effect of glycerol in this case was some specific effect on soybean trypsin inhibitor (Kunitz) or a general effect on peptide bond hydrolysis equilibria. In this

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paper we show, by measuring equilibrium constants for simple blocked dipeptides, that a large part (but not all) of the effect is general. We proceed to show that further increases in concentrations of cosolvents to approximately 80% (v/v) cause further significant decreases in K_{hyd}. Comparison of the effects of organic cosolvents on $K_{syn} = 1/K_{hyd}$ with their effect on the pK values of the COOH and NH_2 termini of the reactants leads us to conclude that the major cause of the organic cosolvent effect is the decrease in the equilibrium constant for the transfer of the proton from the COOH terminus to the NH_2 terminus of the reactants. Finally we apply the system to the enzymatic addition of L-methionineamide to the COOH termini of several small peptides. Such an addition may prove useful since it should allow for the coupling of any enzymatically prepared peptide via the homoseryllactone method of Horn & Laursen (1973).

The notion of adding organic cosolvents to shift equilibria in enzyme-catalyzed reactions toward synthesis is not unique to our laboratory. Ingalls et al. (1975) described an increased synthesis of esters by chymotrypsin and elastase in 80% alcohol. Butler & Reithel (1977) obtained significant urease-catalyzed syntheses of urea in the presence of large concentrations of organic cosolvents.

Nomenclature and General Considerations

In this paper for protein substrates, we retain the designation K_{hyd}, employed in previous papers from our laboratory, for the equilibrium constant for peptide bond hydrolysis. For cyclic substrates this is defined as

$$K_{hyd} = \frac{\Sigma(\text{nicked})}{\Sigma(\text{intact})} \quad (1)$$

where the parentheses designate concentrations, nicked refers to the protein with one *specified* peptide bond hydrolyzed, and intact refers to the protein with all of its peptide bonds intact. The sums are taken over the various protonated or liganded forms of the protein that coexist at equilibrium. The value of K_{hyd} for cyclic peptide bonds clearly does not depend upon the choice of concentration units used for nicked and intact protein forms; however, it does depend upon pH and solvent composition.

In cases where the hydrolysis of one specified peptide bond produces two fragments, K_{hyd} is given by

$$K_{hyd} = \frac{\Sigma(\text{fragment}_1)\Sigma(\text{fragment}_2)}{\Sigma(\text{intact})} \quad (2)$$

In this case the value of K_{hyd} clearly depends upon the choice of concentration units. We always use molarity for this purpose.

In the work with small peptides, we prefer to use K_{syn}, the inverse of K_{hyd}, and eq 1 becomes

$$K_{syn} = 1/K_{hyd} = \frac{\Sigma(\text{intact})}{\Sigma(\text{nicked})} \quad (3)$$

and eq 2 becomes

$$K_{syn} = 1/K_{hyd} = \frac{\Sigma(\text{intact})}{\Sigma(\text{fragment}_1)\Sigma(\text{fragment}_2)} \quad (4)$$

This emphasizes the *increases* in K_{syn} achieved by addition of organic cosolvents. Had we used K_{hyd} the peak in Figure 5 would have been compressed so extremely that the large change in K_{syn} would be difficult to appreciate.

K_{syn} and K_{hyd} depend upon pH. For simple blocked dipeptides the dependence was stated by Dobry et al. (1952) and the K_{syn} of eq 4 is given by

$$K_{syn} = \frac{K_{syn}^{\circ}}{1 + (H^+)/K_1 + K_2/(H^+)} \quad (5)$$

where K_1 is the ionization constant of the carboxyl group of fragment$_1$ and K_2 is the ionization constant of the α ammonium group of fragment$_2$. For proteins the pH dependence is at least as complex as that of eq 5, but, if peptide bond synthesis leads to perturbation of some side chain ionization constants, additional terms must be included (Mattis & Laskowski, 1973).

The synthesis of a peptide bond leads to elimination of a water molecule from the reactants. Yet, following the usual conventions, eq 1–4 do not include water concentration. This is of considerable practical advantage in calculating the expected thermodynamic yields of synthesized peptide.

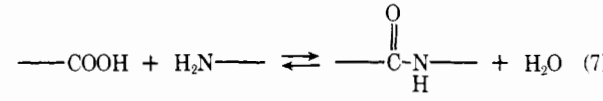
However, when we inquire into why K_{syn} rises when an organic cosolvent is added, water must be included into the equilibrium expression since the natural (but incorrect) conclusion is that the increase in K_{syn} is a *direct* result of reduction in water activity. Most of the cosolvents used in this work are essentially nonvolatile (in comparison with water). Therefore water activity can be readily measured as the ratio of the vapor pressure of the solution, P_{H_2O}, divided by the vapor pressure of pure water, $P_{H_2O}^{\circ}$. In order to utilize this ratio we define

$$\S_{syn} = \frac{(P_{H_2O}/P_{H_2O}^{\circ})X_{product}}{X_{fragment_1}X_{fragment_2}} \quad (6)$$

where X denotes the mole fraction.

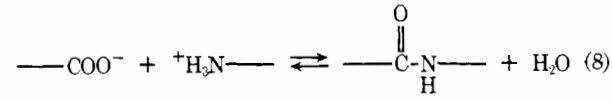
Were the *direct* reduction in water activity solely responsible for the increase in K_{syn} (eq 4), then $\S_{syn}$ should be approximately independent of the added organic cosolvent. As is shown in this paper this is clearly not so and an additional reason for the increase in K_{syn} must be found.

Carpenter (1960) introduced a conjecture that peptide bond synthesis equilibria are independent of substituent effects if the equilibrium constant for the synthesis is defined in terms of classical forms of the reactants rather than in terms of ionized forms. He stated that the equilibrium,



(7)

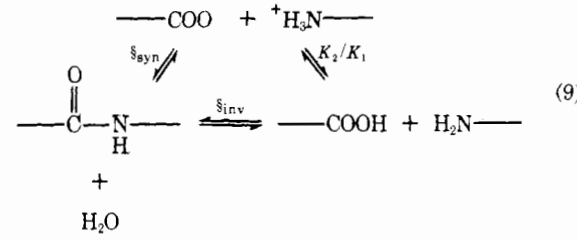
where the lines denote peptide fragments 1 and 2, is independent of the presence of charged groups on the remainder of the peptide, while the commonly written equilibrium (Dobry et al., 1952)



(8)

significantly depends upon the presence of such charged groups. His conclusion is in excellent accord with the very small amount of thermodynamic data on peptide bond synthesis available in the literature.

We have therefore extended his assumption to the effect of organic cosolvents and generated a scheme:



(9)

This cycle leads directly to the equation

$$\S_{syn} = \S_{inv}K_2/K_1 \quad (10)$$

where $\S_{inv}$ is independent of solvent composition according to our extension of the Carpenter (1960) conjecture. Taking logarithms leads to

$$\log \S_{syn} = \log \S_{inv} - (pK_2 - pK_1) = \log \S_{inv} - \Delta pK \quad (11)$$

where ΔpK is the difference between the pK_2 of the ammonium and the pK_1 of the carboxyl groups in the reactants. Upon addition of organic cosolvent, the value of the proton transfer equilibrium constant, K_1/K_2, decreases (see Table I). This change is measured by the quantity $\Delta\Delta pK$, defined by:

$$\Delta\Delta pK = (\Delta pK)_{water} - (\Delta pK)_{cosolvent}$$
$$= (pK_2 - pK_1)_{water} - (pK_2 - pK_1)_{cosolvent} \quad (12)$$

If we are interested only in the change in $\S_{syn}$ as a function of addition of organic cosolvent, eq 11 becomes:

$$\Delta \log \S_{syn} = \Delta\Delta pK \quad (13)$$

It should be pointed out that none of the above is thermodynamically rigorous. K_{hyd} and K_{syn} employ concentrations and not activities. While $\S_{syn}$ is a hybrid where the water activity, $P_{H_2O}/P_{H_2O}°$, is properly used, the other entries are mole fractions and not activities. In fact it is pointed out later in this paper that the value of K_{syn} depends upon the concentration of glycineamide employed in synthesis, a clear indication that the activity of glycineamide is not directly proportional to concentration over the range employed. Thus *all* the equilibrium constants used here carry an implied subscript K_{app} and all the conclusions based on their manipulation may be flawed by lack of rigor.

Experimental Procedures

Materials

Bovine α-chymotrypsin (EC 3.4.21.1) (3× crystallized and salt free), glycineamide hydrochloride, L-methionineamide hydrochloride, glycylglycyl-L-phenylalanine, glycyl-L-leucyl-L-tyrosine, *N*-acetylglycine, hippuric acid, Mes[1] (2-(*N*-morpholino)ethanesulfonic acid)), glycyl-L-lysine, benzoyl-L-arginine, and bradykinin triacetate were purchased from Sigma Chemical Co. Porcine trypsin (EC 3.4.21.4) was obtained from Worthington Biochemical Corp. Vega-Fox Chemicals supplied *N*-Cbz-L-tryptophan and *N*-Cbz-L-tryptophanylglycineamide. Analytical grade acetonitrile and spectral grade dioxane were supplied by Mallinckrodt. Analytical grade glycerol was from Baker. Aldrich supplied triethylene glycol, 1,5-pentanediol, and analytical grade ethylene glycol. Eastman Organic Chemicals supplied practical grades of 1,4-butanediol and 1,3-propanediol. The practical grade solvents were not repurified prior to use nor was a rigorous exclusion of water attempted. The Waters C_{18} μ-Bondapak column was used for high-performance liquid chromatography.

Procedures

High-performance liquid chromatography was used to separate substrate and product so their ratios could be monitored as they approached equilibrium in the organic cosolvent mixtures. The extreme sensitivity and capability for routine separations allowed detection of less than 0.1 nmol of peptide in less than 15 min. In our system the elution was monitored spectrophotometrically at 254 nm; therefore, chromophoric substrates and/or products were employed. Routine assays depended on the capability of separating *N*-Cbz-L-tryptophan and *N*-Cbz-L-tryptophanylglycineamide on a Water Associates C-18 μ-Bondapak column which was run reverse phase, and thermostated from 40 to 45 °C with thermal tape. The column solvent was pumped by a Waters Model 6000A chromatography pump (0.5–1.5 mL/min) and the absorbance was monitored by a Waters Model 440 absorbance detector (0.005–0.05 Å full scale) and recorded and integrated on a Houston Instruments Omniscribe recorder. The compounds were repurified by high-performance liquid chromatography prior to use because of chromophoric contaminants which otherwise interfered with quantitation. For the purification, *N*-Cbz-L-tryptophan (16 mg/mL) or *N*-CBz-L-tryptophanylglycineamide (16 mg/mL) was dissolved in 50% acetonitrile–50% water and eluted with the same solvent at 1.5 mL/min (column temperature, 45 °C). Up to 4 mg of peptide in 2.5 mL could be purified in a 15-min elution. The purified compounds were then concentrated to dryness in a rotary evaporator.

When glycineamide is condensed with Cbz-L-tryptophan, the product, *N*-Cbz-L-tryptophanylglycineamide, is more hydrophobic. Conditions for elution of the two compounds were sought so that the separation was complete, the separation could be accomplished with a minimal elution time, and components of interest did not elute in or near the void volume. Inclusion of salt in the solvent was essential for separation. The solvent used was 20% (v/v) acetonitrile–80% (v/v) 10 mM Mes, 10 mM NaH_2PO_4, pH 6.6. Over the substrate range routinely used, 1–10 nmol, the quantitation (integrated peak area) was proportional to the amount injected, and the cochromatography of Cbz-L-tryptophan and Cbz-L-tryptophanylglycineamide in different ratios to each other did not interfere with the quantitation of either. With the conditions used for assay, anomolous peaks were observed in the void volume (see Figure 2). Chromatography of recrystallized 0.1 M glycineamide hydrochloride or of 0.1 M sodium acetate also produced these peaks.

For each assay, 10-μL aliquots which contained chymotrypsin (120 μg per aliquot) were injected onto the column. Protein sticks to the column, reducing the resolution of the column and increasing the back pressure. Consideration was given to deproteinizing the samples by ultrafiltration or other means; however, this would have reduced the precision of the assay. In this work, at least 3000 chromatograms (~360 mg of protein) were run with the only precaution of weekly flushings with 1 L of 50% acetonitrile, followed by 1 L of 25% acetonitrile–75% column buffer containing 1% sodium dodecyl sulfate and followed by 1 L of 50% acetonitrile. The back pressure of the column eventually rose from 1500 psi at 1.5 mL/min so the flow was slowed gradually to 0.5 mL/min and the temperature of the jacketed column was increased from 45 to 53 °C to keep the back pressure below 3000 psi.

Assay of Equilibrium Positions. The principle of the assay was to incubate 1 mM Cbz-L-tryptophan, 0.1 M glycineamide, and chymotrypsin (2 mg/mL) in the appropriate cosolvent and to determine α, the fraction of dipeptide, at various time intervals until equilibrium was reached. Similarly in the direction of hydrolysis, 1 mM Cbz-L-tryptophanylglycineamide was incubated with 0.1 M glycineamide and chymotrypsin (2 mg/mL). Since equilibrium was approached from both directions, proof of equilibrium was provided in each case and extrapolation to equilibrium from only one direction was

[1] Abbreviations used: Mes, 2-(*N*-morpholino)ethanesulfonic acid; Cbz, benzyloxycarbonyl; K_{hyd}, K_{syn}, and $\S_{syn}$ are defined by eq 2, 4, and 6, respectively; app, apparent.

avoided. To prepare the substrates, 13.5 mg of purified Cbz-L-tryptophan or 15.8 mg of purified Cbz-L-tryptophanylglycineamide was dissolved in 1.0 mL of acetonitrile or 1.0 mL of 50% acetonitrile–50% water, respectively. Aliquots (25 μL) of each solution were injected into twist cap vials and allowed to dry. Pure cosolvent (0–0.8 mL) was added to the vials 1 day prior to use to allow for slow solubilization. Then to each of two vials, one prepared for synthesis and to one prepared for hydrolysis, 100-μL aliquot of a stock solution of 1.0 M glycineamide in water, adjusted to pH 6.7 was added, followed by a 100-μL aliquot of chymotrypsin (20 mg/mL) in water, pH 6.7. The final volume was 1.0 mL and the concentrations were 1 mM in either Cbz-L-tryptophan or Cbz-L-tryptophanylglycineamide, 0.1 M glycineamide, and 2 mg/mL chymotrypsin. The choice of 6.7 for the pH(app) of the incubation mixture was made since K_{syn} is near its maximum at this pH value. Chymotrypsin was added dissolved in water because of the difficulty of dissolving the crystals directly in partially nonaqueous solvents (Singer, 1962).

The reaction was considered impractical with a particular cosolvent if equilibrium was not attained in 2 months and the studies were then discontinued. When the α(s) obtained from both directions of hydrolysis and synthesis agreed to within 5% the equilibrium position was further calculated by extrapolation.

Since most of the cosolvents used were alcohols, the possibility of ester formation was considered; however, no additional peaks were detected. In order to exclude the possibility of ester cochromatographing with Cbz-L-tryptophanylglycineamide, Cbz-L-tryptophan was incubated with chymotrypsin in the absence of glycineamide and in the presence of glycerol or 1,4-butanediol to verify that no new peak formed.

Effect of pH on K_{syn} was investigated to check our model and for practical considerations. Stock solutions of 1 mM Cbz-L-tryptophan or 1 mM Cbz-L-tryptophanylglycineamide, 0.1 M glycineamide, and 2 mg/mL chymotrypsin in 60% (v/v) of one of the favorable cosolvents at pH(app) 6.7 were halved. Half the volume was adjusted down in pH(app) and at each 0.5 pH increment, 1.0 mL was withdrawn into a vial. The same was done for the other half as its pH(app) was adjusted upward. The operation was repeated for a 100% aqueous incubation mixture except 1.0 M glycineamide was substituted for 0.1 M to increase the α values and hence the accuracy of their determinations.

Apparent pK(s) of the peptide substrates glycineamide and Cbz-L-tryptophan in different cosolvents at different cosolvent concentrations were determined to investigate whether the shift in equilibrium was due quantitatively to the pK perturbation. An attempt was made to titrate the compounds at the same concentrations as used for their enzyme-catalyzed condensation, 0.1 M for glycineamide, and 1 mM for Cbz-L-tryptophan. However, because the dissociation of water masked the titration of 1 mM Cbz-L-tryptophan at low cosolvent concentrations, where the pK(app) was lowest, only at higher cosolvent concentrations could pK(app)(s) be determined. Also, since Cbz-L-tryptophan is insoluble in water at concentrations much greater than 1 mM, the more soluble *N*-acetylglycine and hippuric acid at 0.1 M were titrated instead, on the assumption that their pK shifts would be the same as for 1 mM Cbz-L-tryptophan. The potentiometric titrations were performed on a Radiometer Titrator TTT 2 equipped with pen drive REA 300, recorder REA 110, and Autoburette ABU 13 (0.25-mL capacity). The electrodes used were Radiometer calomel electrode K 4112 and glass electrode G2222 C. Prior to use the electrodes were soaked in the appropriate cosolvent mixture for 15 min at 25 °C. Solutions (3.0 mL) of the peptides were titrated under N_2 (occasionally N_2 was bubbled through the highly viscous solutions to aid in stirring) with 2 N NaOH for 0.1 M glycineamide or with 2 N HCl for 0.1 M *N*-acetylglycine or 0.1 M hippuric acid or with 0.02 N HCl for 1 mM Cbz-L-tryptophan. The dilution by titrant was considered insignificant. The compounds were titrated as their sodium or chloride salts while thermostated at 25 °C. Higher concentrations of neutral salts ordinarily added to keep the ionic strength constant were not added because of the stabilizing effect on the ionized forms in partially nonaqueous solvents and because the conditions were chosen so as to mimic the conditions for synthesis. The pK(app)(s) were determined with a precision of ±0.04 unit.

The absolute values of the pK(s) are of only limited significance since the pH meter was calibrated only in water, and therefore it is not clear what the pK(s) mean. However, differences between pK(s) of two acids obtained in the same solvent are meaningful provided only that the Nernst equation is still applicable in the mixed solvents. This was proven to be correct for water–ethylene glycol mixtures by Sage & Singer (1962).

Enzyme-catalyzed addition of L-methionineamide onto the carboxyl terminal was attempted to demonstrate the possibility and practicality of this method. This was attempted with Gly-Gly-L-Phe and Gly-L-Leu-L-Tyr as chymotryptic substrates or with benzoyl-L-arginine, Gly-L-Lys, or bradykinin as tryptic substrates. The peptide (10 or 2 μmol for bradykinin only) was first dissolved in 50 μL of water; 0.85 mL of 1,4-butanediol was then added, followed by the addition of enough solid L-methionineamide for a final concentration of 0.1 M. After the L-methionineamide was completely dissolved (up to 2 h), 0.1 mL of a 30 mg/mL trypsin solution containing 10 mM $CaCl_2$ or 0.1 mL of a 20 mg/mL chymotrypsin solution in water was added. The pH(app) was then adjusted to 6.7. In separate experiments glycineamide was substituted for L-methionineamide to demonstrate the generality of the approach. After 10 days for the chymotrypsin reaction or 3 weeks for the trypsin reaction, aliquots (100 μL) of the mixtures were applied to 15-cm wide Whatman 3MM paper strips and the strips were subjected to electrophoresis for 60 min at 3 kV in pH 6.5 pyridine–acetate buffer (Bennet, 1967) on a Gilson Model D high voltage electrophorator. The bands were located by spraying with ninhydrin (1% in ethanol) or with Sakaguchi reagent (Bennet, 1967) for the arginyl peptides. The peptides were then eluted with 0.01 N formic acid and the washes concentrated on a rotary evaporator. From amino acid analyses on a Durrum D-500 amino acid analyzer, the fraction of substrate condensed with L-methionineamide was determined.

Results

Effect of 60% (v/v) Glycerol on K_{hyd} in Soybean Trypsin Inhibitor (Kunitz). Figure 1 presents the comparison between the attainment of virgin ⇄ modified inhibitor equilibrium (Arg^{63}-Ile peptide bond intact ⇄ bond hydrolyzed) in soybean trypsin inhibitor (Kunitz) in water and in 60% (v/v) glycerol. These data were obtained by withdrawing aliquots of incubation mixtures with bovine trypsin and analyzing for reactants and products by disc gel electrophoresis. The Methods and Materials for this section are described in detail by Mattis & Laskowski (1973). Inspection of Figure 1 shows a dramatic eightfold decrease in K_{hyd} as a result of 60% (v/v) glycerol addition. It is clear that an effect much greater than expected from the trivial decrease of water concentration to 40% is seen. Such an effect could be either some specific effect on the conformation or side chain ionization constants of soybean trypsin inhibitor (Kunitz) at pH 5 or a general effect of ther-

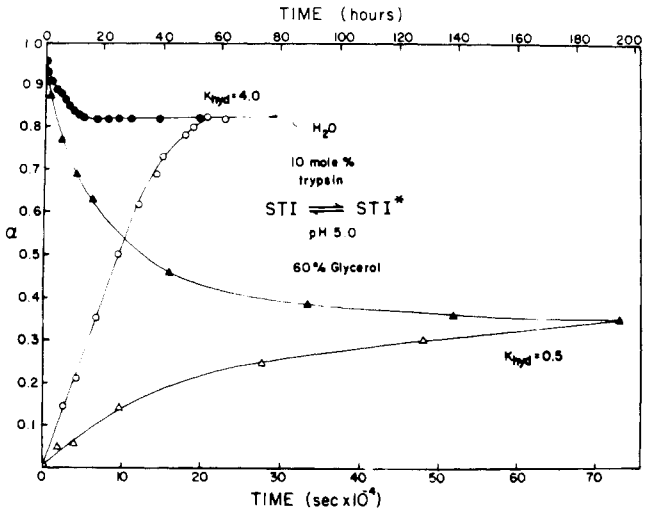


FIGURE 1: The dependence of the fraction, α, of modified (Arg^{63}-Ile peptide bond hydrolyzed) soybean trypsin inhibitor (Kunitz) upon the time of incubation with bovine β-trypsin. The reaction was catalyzed by 10 mol % of trypsin in 0.50 M KCl–0.05 M $CaCl_2$–0.05 M potassium acetate at pH 5. Forward (○) and reverse (●) run in water; forward (△) and reverse (▲) run in 60% (v/v) glycerol.

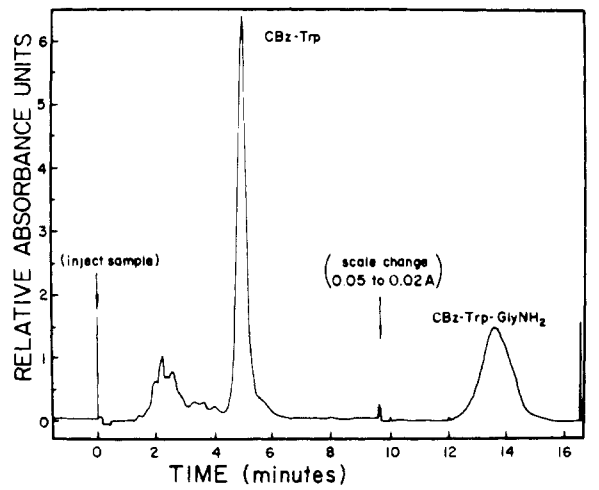


FIGURE 2: High-performance liquid chromatograph of a 10-μL aliquot of a reaction mixture in 60% (v/v) ethylene glycol, pH(app) 6.7, 25 °C, 2 mg/mL chymotrypsin; initial concentration of CbzTrp was 1 mM and of glycineamide was 100 mM; time of incubation was 1 week. The column was eluted with 20% (v/v) acetonitrile–80% (v/v) 10 mM Mes, 10 mM NaH_2PO_4, pH 6.6 at 40 °C. Absorbance was monitored at 254 nm. The anomalous peaks at void volume are attributed to the high salt concentration.

modynamic stabilization of peptide bonds in the presence of organic cosolvents. The rest of this paper demonstrates that the effect is largely general.

The Cbz-L-tryptophanylglycineamide ⇌ Cbz-L-tryptophan + Glycineamide Equilibrium. This chymotrypsin-catalyzed reaction was monitored by high-performance liquid chromatography. The nonchromophoric $GlyNH_2$ was present in excess and the distribution between Cbz-L-tryptophanylglycineamide and Cbz-L-tryptophan was monitored.

The high-performance liquid chromatograms were complicated by the peaks in the void volume which became more exaggerated as the organic cosolvent concentration increased. However, the peaks did not interfere with the quantitation, as seen in Figure 2, which shows the elution of the components of a sample of 60% (v/v) ethylene glycol and represents one of the more complicated profiles. As a control, Cbz-L-tryptophan was incubated with chymotrypsin in the absence of glycineamide in water or in 80% (v/v) glycerol or in 80% (v/v) 1,4-butanediol at pH(app) 6.7 and no new peaks appeared. This confirms the assignment of the Cbz-L-tryptophanylglycineamide peak and furthermore suggests that there is no significant ester formation between Cbz-L-tryptophan and the cosolvents employed.

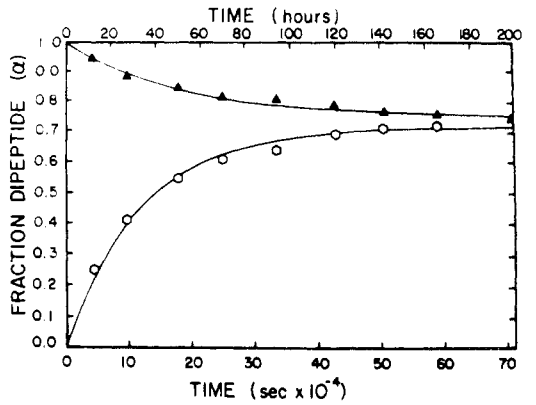


FIGURE 3: The time dependence of the fraction, α, of $CbzTrpGlyNH_2$ upon the time incubation with bovine chymotrypsin. The reaction was catalyzed 2 mg/mL of chymotrypsin in 80% (v/v) 1,4-butanediol, at pH(app) 6.7. Synthesis of $CbzTrpGlyNH_2$ from 1 mM CbzTrp and 100 mM $GlyNH_2$ (○), hydrolysis of 1 mM $CbzTrpGlyNH_2$, in presence of 100 mM $GlyNH_2$ (▲). The lines are theoretical for a first-order approach to equilibrium.

Approach to equilibrium was monitored for the most favorable cosolvents. Figure 3 shows that the approach to equilibrium from directions of hydrolysis of Cbz-L-tryptophanylglycineamide (in presence of glycineamide) and of synthesis of Cbz-L-tryptophanylglycineamide from CBz-L-tryptophan and glycineamide in 80% (v/v) 1,4-butanediol containing 2 mg/mL chymotrypsin. In this experiment a colloidal dispersion of enzyme formed in minutes and remained dispersed during the week of equilibration. In 70% (v/v) cosolvent a lighter enzyme dispersion formed in hours and at 60% (v/v) cosolvent a very light dispersion became obvious only after several days.

The rate of synthesis is proportional to the enzyme concentration. Reaction mixtures allowed to incubate for 7 days showed α values of 0.138 at 0.1 mg/mL of chymotrypsin, 0.232 at 0.2 mg/mL, and 0.446 at 0.5 mg/mL.

At cosolvent concentration higher than 50% (v/v), no significant synthesis could be demonstrated in 2 months in ethanol, dimethyl sulfoxide, dimethylformamide, acetone, dioxane, and acetonitrile due to inactivation of chymotrypsin. On the other hand, synthesis proceeded in the various polyhydroxy alcohols at cosolvent concentrations up to 80–85% (v/v).

While detailed kinetic studies were not carried out we have determined half-times for attainment of equilibrium in 0, 20, 40, 60, and 80% (v/v) 1,4-butanediol at pH(app) 6.7, 1 mM chromophoric substrate or product, 100 mM glycineamide, and 2 mg/mL chymotrypsin. They are 5.0, 7.5, 10, 15, and 23 h, respectively. They are roughly the same in the forward and reverse directions as expected from pseudo-first-order reversible reactions. Since addition of 1,4-butanediol increases both K_{syn} and the half-life of attainment of equilibrium, there is a striking compensation—the initial rate of synthesis in all the solutions above was approximately 0.030 mM/h.

Effect of Cosolvent on K_{syn}. Figure 4 shows a semilogarithmic plot of the experimental values of K_{syn} (refer to eq 4) vs. volume % cosolvent for different cosolvents.

It is seen that, for each cosolvent, the logarithm of K_{syn} is

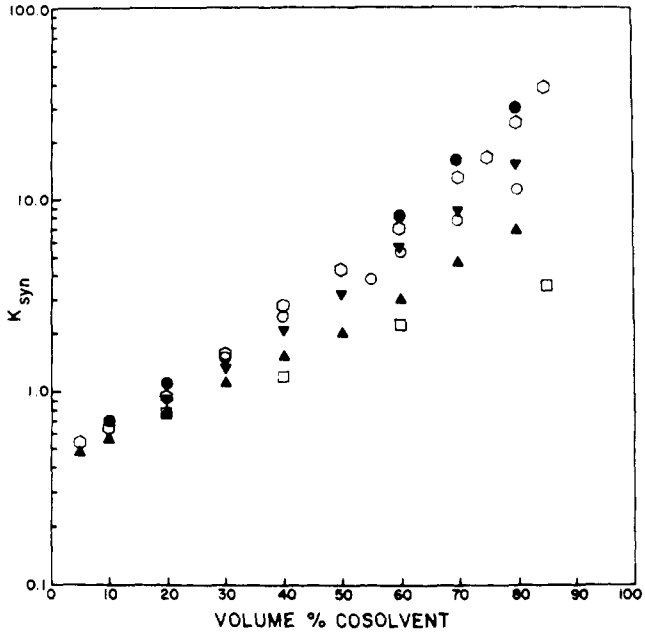


FIGURE 4: The dependence of K_{syn} upon the volume % of cosolvent, glycerol (□), ethylene glycol (▲), 1,3-propanediol (○), triethylene glycol (▼), 1,4-butanediol (○), and 1,5-pentanediol (●). Note that logarithmic spacing is used for the K_{syn} axis.

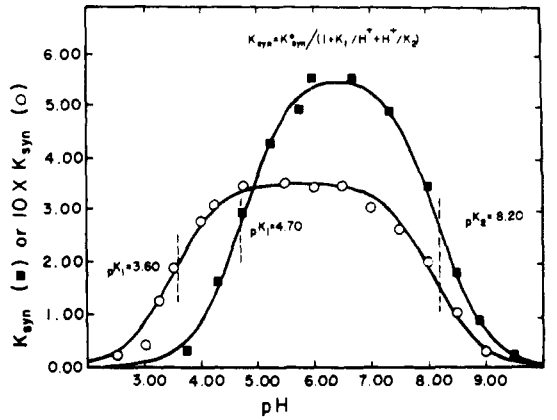


FIGURE 5: The pH dependence of K_{syn}. K_{syn} in water (○); $K_{syn}/10$ in 60% triethylene glycol (■). The curves are best fits of the data to eq 5. The pK values stated on the figure were obtained by potentiometric titration and agree within ±0.05 with those generated by the fit.

linearly related to the volume fraction of this cosolvent. All of the data extrapolate to K_{syn} of 0.45 M^{-1} in water (this value is difficult to determine directly in 100 mM glycineamide). The value of K_{syn} in 60% (v/v) glycerol is 2.12. Thus K_{syn} in 60% (v/v) glycerol is 4.7 times greater than in water. This accounts for most, but not all, of the effect of 60% (v/v) glycerol on K_{hyd} of soybean trypsin inhibitor (Kunitz). From the point of view of practical utility, the most interesting result in Figure 4 is the largest attained increase in K_{syn}. This is an 80-fold increase up to 38 M^{-1} in 85% (v/v) 1,4-butanediol.

Effect of pH on K_{syn} both in water and in 60% (v/v) triethylene glycol is shown in Figure 5. In both cases there is excellent agreement with the equation proposed by Dobry et al. (1952), eq 5. Furthermore the pK values deduced from fitting of K_{syn} as a function of pH agree within 0.05 unit with the pK values determined directly by potentiometric titration. These pK values are marked on the figure. Note the pronounced, expected shift in pK_1 (the pK of the carboxyl group in Cbz-L-tryptophan) upon changing the solvent and essentially no change in pK_2 (the pK of the $-NH_3^+$ of the protonated glycineamide). It is well known that pK(s) of groups whose ionization involves separation of charge (e.g., carboxyl) are greatly influenced by the solvent medium (Michaelis & Mizutani, 1925), while pK(s) of groups whose ionization is a charge transfer (e.g., alkylammonium) are largely unaffected (Mizutani, 1925; Halford, 1933). The increase in pK_1 in 60% (v/v) triethylene glycol is responsible for the great steepening of the K_{syn} vs. pH curve in this cosolvent. It should also be noted that K_{syn} values are much larger in 60% (v/v) triethylene glycol (K_{syn}° = 5.5 M^{-1}) than in water (K_{syn}° = 0.33 M^{-1}). Note that for experimental convenience the value in water was determined in 1 M glycineamide rather than in 100 mM and this accounts for the difference from K_{syn}° = 0.45 M^{-1} obtained by extrapolation of the data in Figure 4. Presumably this is due to the decrease in the activity coefficient of glycineamide when its concentration is changed from 100 mM to 1 M.

Effect of salt concentrations upon K_{syn} is quite pronounced. It should be remembered that our K_{syn} is a concentration constant and therefore the added salt affects K_{syn} by affecting activity coefficients of reactants and products. In 60% (v/v) triethylene glycol K_{syn}° was progressively decreased from 5.5 to 2.7 M^{-1} by raising the concentration of NaCl from 0 to 0.75 M. As a practical consideration, *additional* salt (note that glycineamide hydrochloride is a salt) should be avoided to maximize the yield in syntheses.

Effect of Organic Cosolvent on $\Delta\Delta pK$. We have proposed in the Nomenclature and General Considerations section that the major reason for the increase in K_{syn} upon addition of organic cosolvent (Figures 4 and 5) is the decrease in the equilibrium constant for the transfer of a proton from the -COOH to the $-NH_2$ group in the reactants. The pK values, the logarithms of the equilibrium constant for proton transfer (ΔpK), and the change in this logarithm between water and organic solvent are listed in Table I. Note again the large expected effect on the pK_1 of the carboxyl group, while the pK_2 of the alkylammonium group is little affected. While this is a very satisfactory result, it should be regarded with caution since the absolute meaning of pK(s) obtained in the cosolvent mixtures is questionable. On the other hand, ΔpK and $\Delta\Delta pK$ remain valid since pK_1 and pK_2 are determined in the same solvent, thus eliminating any errors due to improper pH meter calibration.

The $\Delta\Delta pK$ values in 1,4-butanediol–water mixtures were also measured as a function of volume fraction of the cosolvent. Some representative data are also listed in Table I.

Correlation of Δ Log § and of $\Delta\Delta pK$. Figure 6 is a plot of Δ log § vs. $\Delta\Delta pK$. The simple model developed in Nomenclature and General Considerations predicts that this should be a straight line of unit slope (eq 13). It is seen in Figure 6 that, for the lower data points (i.e., over the 0–60% (v/v) 1,4-butanediol concentration range), the fit is excellent, strongly supporting our model. On the other hand, above this concentration range, the increase in Δ log § is much less than expected from our prediction. Presumably another effect upon the activity coefficients of reactants and/or products is responsible for this effect. We have no simple explanation for this unfortunate (since it makes K_{syn} lower than it would be otherwise) effect. Unfortunately, because of lack of sufficient enzymatic activity in other cosolvents our model could be tested only with polyhydroxy compounds.

Enzymatic addition of L-methionineamide onto the carboxyl termini of several different substrates was successful. Verification of the condensation of substrate with L-methionineamide was based on the presence of methionine in the amino acid compositions of the purified products. These

TABLE I: Effect of Cosolvent on pK.

cosolvent	(% v/v)	app[a] pK_1 of N-acetylglycine	app pK_2 of glycineamide	ΔpK $(pK_2 - pK_1)_{cosolvent}$	$\Delta\Delta pK$ $(\Delta pK_{H_2O} - \Delta pK_{cosolvent})$
(water)	(0)	3.60	8.20	4.60	0.00
glycerol	(80)	4.48	8.20	3.72	0.88
ethylene glycol	(80)	4.83	8.20	3.37	1.23
1,3-propanediol	(80)	4.96	8.20	3.14	1.36
triethylene glycol	(80)	5.10	8.32	3.02	1.38
ethanol	(80)	4.95	8.02	3.07	1.53
acetonitrile	(80)	5.00	8.05	3.05	1.55
1,5-pentanediol	(80)	5.10	8.15	3.05	1.55
acetone	(80)	5.49	8.00	2.51	2.09
dioxane	(80)	5.53	8.00	2.47	2.13
dimethyl sulfoxide	(80)	6.93	8.10	1.11	3.49
1,4-butanediol	(5)	3.70	8.20	4.50	0.12
	(10)	3.76	8.20	4.44	0.16
	(20)	3.93	8.20	4.27	0.33
	(30)	4.09	8.20	4.11	0.49
	(40)	4.20	8.20	4.00	0.60
	(50)	4.40	8.20	3.80	0.80
	(60)	4.53	8.17	3.64	0.96
	(70)	4.83	8.15	3.32	1.28
	(80)	5.10	8.15	3.05	1.55
	(85)	5.30	8.10	2.80	1.80

[a] app, apparent.

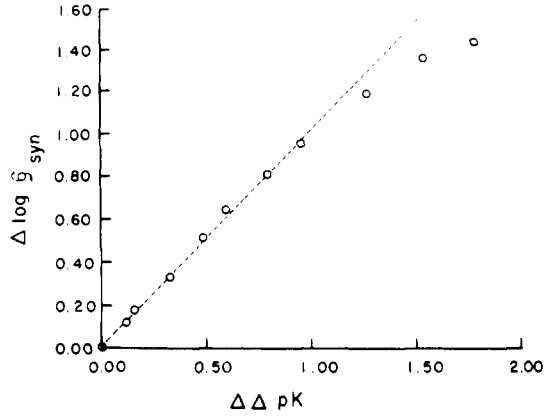


FIGURE 6: The dependence of the logarithm of the synthesis equilibrium constant $\S_{syn}$ (defined by eq 6) upon the change in the difference between the pK values of the alkylammonium and carboxyl groups of the reactants, $\Delta\Delta pK$ (defined by eq 12). The dashed line is drawn with a unit slope as predicted by eq 13.

analyses showed variations in the residue composition of less than 0.03 residue. The yield was based on the fraction of methionine-containing product of the total peptide material eluted from the chromatography paper. The recovery of total peptide from the paper was routinely 50% of the starting material. The percentage of product for the substrate Gly-L-Leu-L-Tyr was 52%; for Gly-Gly-L-Phe, 70%; for benzoyl-L-arginine, 49%; for Gly-L-Lys, 85%; and for bradykinin, 50%. When Gly-L-Lys and $GlyNH_2$ were incubated with trypsin 85% 1,4-butanediol, the yield of tripeptide was 40%, showing that the favorable yields were not limited to the use of L-methionineamide. It should be noted that these syntheses were not necessarily at equilibrium. The substrate Gly-L-Leu-L-Tyr could potentially be hydrolyzed to Gly-Leu and free tyrosine. This was not observed nor did we detect any L-methionineamide adducts other than Gly-L-Leu-L-Tyr-L-$MetNH_2$.

Discussion

In this paper we show that addition of large amounts of organic cosolvents significantly increases the value of K_{syn} for synthesis of peptide bonds. Unfortunately, such an addition generally decreases the solubility of the reactants and possibly even more importantly decreases the solubility, stability, and catalytic efficiency of the enzyme required for the attainment of equilibrium. For bovine chymotrypsin and our test equilibrium of Cbz-L-tryptophanylglycineamide formation the best compromise system was 85% (v/v) 1,4-butanediol where K_{syn} rises to 38 M^{-1} from 0.45 M^{-1}. This 80-fold change in K_{syn} should make enzymatic synthesis of several specialized bonds in peptides and proteins quite practical. We provide here an illustration by carrying out trypsin and chymotrypsin catalyzed addition of L-methionineamide to COOH termini of several peptides. We anticipate that, after exhaustive hydrolysis of a protein by some endopeptidase, the solvent can be altered to 85% (v/v) 1,4-butanediol, the enzyme concentration raised, and excess L-methionineamide added. After complete inhibition of the enzyme, the resultant peptides with L-methionineamide at the termini can be separated and attached to insoluble supports by the CNBr method of Horn & Laursen (1973) for subsequent solid phase sequencing. We can envisage several other applications of the present system with K_{syn} 80 times greater than K_{syn} in water. However, there are many other possible applications for which the present system is not yet adequate because the increase in K_{syn} is not sufficient or because the rate of synthesis is too slow.

It appears to us that the best route to search for improvements is to study a variety of endoproteinases. The work described here was done with bovine chymotrypsin and with porcine trypsin simply as a matter of convenience. It is quite likely that endopeptidase which better withstands the addition of organic solvent can be found. Bacterial enzymes seem likely candidates.

A problem that caused us considerable concern—the possible loss of enzyme specificity upon addition of organic cosolvent—appears to be much less important than we feared. In the soybean trypsin inhibitor (Kunitz) system in 60% glycerol, trypsin hydrolyzes and synthesizes only a single peptide bond just as it does in water. In the trypsin- and chymotrypsin-catalyzed addition of glycineamide and L-methionineamide, high voltage paper electrophoresis revealed

no other products (although of course some could have escaped detection). Thus while a rigorous study of specificity of endopeptidase in presence of organic cosolvents is very much in order, it can be assumed that the standard specificity is largely retained.

The quantitative part of this work was made possible by the use of high performance liquid chromatography. It seems likely that the reason for the very small number of peptide hydrolysis equilibrium constants at present available in the literature was the lack of a sufficiently rapid quantitative technique. The value of K_{syn} in water obtained in this work 0.45 M^{-1} for Cbz-L-tryptophanylglycineamide compares favorably with K_{syn} of 0.56 M^{-1} obtained by Dobry et al. (1952) for benzoyl-L-tyrosineglycineamide and with K_{syn} of 0.33 M^{-1} for the addition of glycineamide to the COOH-terminal Arg^{63} of modified (Arg^{63}-Ile bond hydrolyzed) soybean trypsin inhibitor (Kunitz) where the NH_2-terminal Ile^{64} was carbamoylated (Wang & Laskowski, 1972; quoted by Kowalski et al., 1974). In making this comparison we should bear in mind that the K_{syn} values obtained in our laboratory are not strictly thermodynamic values but that they were obtained in 0.1 M and 3 M glycineamide, respectively, without making corrections for the nonideality of glycineamide. Nonetheless the three values are similar enough to suggest that the K_{syn} values for addition of glycineamide to a COOH terminus of any peptide or acylamino acid are likely to be closely similar.

We present here an argument that the main cause of increase of K_{syn} upon addition of organic cosolvents is an increase in the pK of the carboxyl group of the reactants or more properly the decrease in the equilibrium constant for the transfer of a proton from the COOH terminus to the NH_2 terminus of the reactants. We must caution again that this argument is not rigorous since only the concentrations and not the activities of the reactants and products (except for water) were measured. Nonetheless we believe that semiquantitatively the argument is correct.

In summary we describe and provide a rationale for a new method of manipulating K_{syn}, which should make enzymatic peptide synthesis and protein semisynthesis more broadly applicable than before, although we are well aware that a truly general method is not yet at hand and that the choice of optimal solvent conditions should still be reinvestigated for each new application.

Acknowledgment

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