Satoru Tokutomi for his cooperation during synthesis of spin labels and to Professor Hiroshi Shimizu for his helpful discussions.

References

Aganoff, B. W., and Suomi, W. D. (1963), *Biochem. Prep. 10,* 47.
Bretscher, M. S. (1973), *Science 181,* 622.
Devaux, P., and McConnell, H. M. (1972), *J. Am. Chem. Soc. 94,* 4475.
Esfahani, M., Limbrick, A. R., Knutton, S., Oka, T., and Wakil, S. J. (1971), *Proc. Natl. Acad. Sci. U.S.A. 68,* 3180.
Hubbell, W. L., and McConnell, H. M. (1971), *J. Am. Chem. Soc. 93,* 314.
Ishinaga, M., and Kito, M. (1974), *Eur. J. Biochem. 42,* 483.
Ito, T., and Ohnishi, S. (1974), *Biochim. Biophys. Acta 352,* 29.
Kornberg, R. D., and McConnell, H. M. (1971), *Biochemistry 10,* 1111.
Linden, C. D., Wright, K. L., McConnell, H. M., and Fox, C. F. (1973), *Proc. Natl. Acad. Sci. U.S.A. 68,* 3180.
Maeda, T., and Ohnishi, S. (1974), *Biochem. Biophys. Res. Commun. 60,* 1509.
Ohnishi, S., and Ito, T. (1973), *Biochem. Biophys. Res. Commun. 51,* 132.
Ohnishi, S., and Ito, T. (1974), *Biochemistry 13,* 881.
Overath, P., Hill, F. F., and Lamnek-Hirsch, I. (1971), *Nature (London), New Biol. 234,* 264.
Papahadjopoulos, D., Poste, G., and Schaeffer, B. E. (1973), *Biochim. Biophys. Acta 323,* 23.
Sanders, H. (1967), *Biochim. Biophys. Acta 144,* 487.
Seimiya, T., and Ohki, S. (1973), *Biochim. Biophys. Acta 298,* 546.
Singer, S. J., and Nicolson, G. L. (1972), *Science 175,* 720.
Singleton, W. S., Gray, M. S., Brown, M. L., and White, J. L. (1965), *J. Am. Oil Chem. Soc. 42,* 53.
Träuble, H., and Eibl, H. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 214.
Verkleij, A. J., de Kruyff, B., Ververgaert, P. H. J. Th., Tocanne, J. F., and van Deenen, L. L. M. (1974), *Biochim. Biophys. Acta 339,* 432.
Waggoner, A. S., Kingzett, T. J., Rottschaefer, S., and Griffith, O. H. (1969), *Chem. Phys. Lipids 3,* 245.
White, D. A. (1973), in Form and Function of Phospholipids, Ansell, G. B., Hawthorne, J. N., and Dawson, R. M. C., Ed., Amsterdam, London, and New York, Elsevier, p 441.
Yang, S. F. (1969), *Methods Enzymol. 14,* 208.

Circular Dichroism and Gel Filtration Behavior of Subtilisin Enzymes in Concentrated Solutions of Guanidine Hydrochloride†

Michael F. Brown‡ and Thomas Schleich*

ABSTRACT: The circular dichroism of diisopropylphosphorylsubtilisins Novo and Carlsberg in both the near- and far-ultraviolet spectral regions is unaltered by concentrations of guanidine hydrochloride as high as 4 *M* at neutral pH. At concentrations of guanidine hydrochloride greater than 4 *M* slow irreversible time-dependent changes, apparently obeying second-order kinetics, are evident in both the near- and far-ultraviolet circular dichroism of these enzymes. Gel filtration studies of inactivated subtilisin enzymes reveal the circular dichroism changes to be accompanied by the appearance of aggregated protein material. The changes in circular dichroism and the production of associated subtilisin species are sensitive to protein concentration, denaturant concentrations, and pH. The circular dichroism of active subtilisins Novo and Carlsberg in guanidine hydrochloride exhibits irreversible changes similar to those observed for the inactivated subtilisins. Aggregated protein material is also formed initially in the presence of guanidine hydrochloride, but is rapidly autolyzed to low molecular weight fragments.

The diisopropyl fluorophosphate sensitive alkaline proteases elaborated by *Bacillus subtilis* and related species are noted for their unusual conformational stability in the presence of biopolymer denaturants. Previous work with the subtilisin enzymes has established that they retain considerable enzymatic activity in concentrated solutions of urea, guanidine hydrochloride (Gdn·HCl),[1] and ethanol. For example, subtilisin Novo[2] is enzymatically active in 6 *M* urea or 50% ethanol, although it is rapidly and irreversibly denatured below pH 5 (Gounaris and Otteson, 1965). Subtilisin Carlsberg is reported to be stable in 10 *M* urea or 6 *M* Gdn·HCl on the basis of optical rotatory dispersion and vis-

† From the Division of Natural Sciences, University of California, Santa Cruz, California 95064. *Received November 6, 1974.* This work was supported by Grant . GB-19503 from the National Science Foundation.

‡ This work constitutes part of a thesis submitted in partial fulfillment of the requirements for the Ph.D. degree at the University of California, Santa Cruz.

[1] Abbreviations used are: Dip-F, diisopropyl fluorophosphate; Dip, diisopropylphosphoryl; $PhCH_2SO_2$, phenylmethanesulfonyl; CD, circular dichroism; uv, ultraviolet; Gdn·HCl, guanidine hydrochloride; Gdn·SCN, guanidine thiocyanate.

[2] Subtilisins Novo and BPN′ are identical enzymes (Olaitan et al., 1968; Robertus et al., 1971; Drenth et al., 1972). We use the name subtilisin Novo when referring to this enzyme.

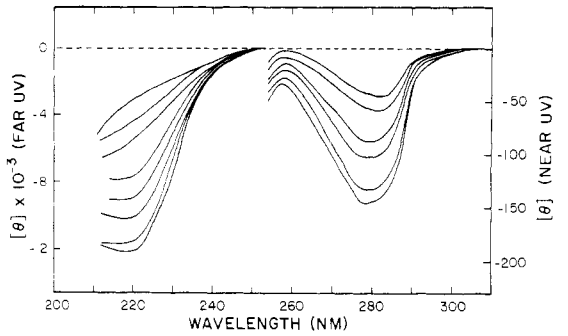

FIGURE 1: The near- and far-uv CD of Dip-subtilisin Novo as a function of time after solution in Gdn·HCl. The sample contained 2.2 mg/ml of protein, 6.99 *M* Gdn·HCl, and 0.04 *M* NaCl at pH 6.3. The near-uv CD was recorded at 10, 20, 50, 70, 145, and 205 min after solution and is presented in order of decreasing absolute amplitude; the far-uv CD was recorded at 10, 20, ca. 30, 50, 70, ca. 100, 145, and 205 min after solution.

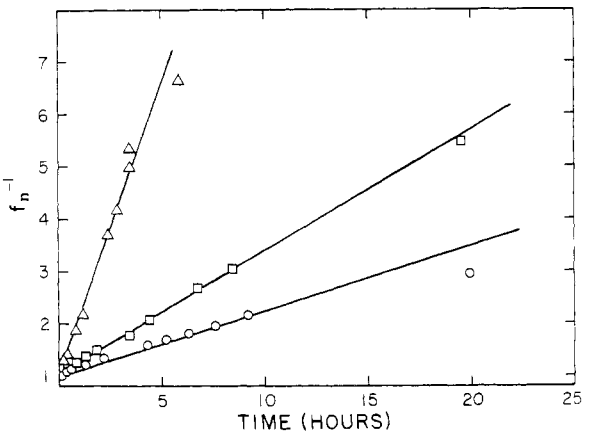

FIGURE 2: The effect of Gdn·HCl concentration on the rate of the spectral changes in the near-uv CD of Dip-subtilisin Novo. The samples contained 2.2 mg/ml of protein and 0.04 *M* NaCl at pH 6.3: 4.05 *M* Gdn·HCl (○); 5.23 *M* Gdn·HCl (□); 6.99 *M* Gdn·HCl (△). f_n was calculated at 278 nm.

cosity measurements (Stauffer and Sullivan, 1971). The stability of subtilisin enzymes in aqueous detergent solutions is well known (Tsuru, 1969; Stauffer and Treptow, 1973).

It is difficult to discern any basis for the unusual stability of subtilisin enzymes from an examination of the amino acid sequence data (Markland and Smith, 1971) or crystal structure of subtilisin Novo (Wright et al., 1969; Drenth et al., 1972). As a first step in a series of studies designed to probe the basis of the exceptional conformational stability of these enzymes we have examined the circular dichroism and gel filtration behavior of diisopropyl fluorophosphate inhibited subtilisins Novo and Carlsberg in concentrated solutions of Gdn·HCl. At concentrations of Gdn·HCl greater than 4 *M* slow irreversible changes occur in the ultraviolet (uv) circular dichroism (CD) of both subtilisins. Gel filtration experiments reveal that these changes in optical activity are accompanied by the appearance of associated material.

Materials and Methods

Protein Samples and Reagents. Crystalline subtilisins Novo (lot no. 120-2) and Carlsberg (lot no. 70-3) were obtained from Novo Industri, Copenhagen, Denmark. Diisopropyl fluorophosphate (Dip-F) was supplied by the Sigma Chemical Co., St. Louis, Mo., and the Gdn·HCl used was

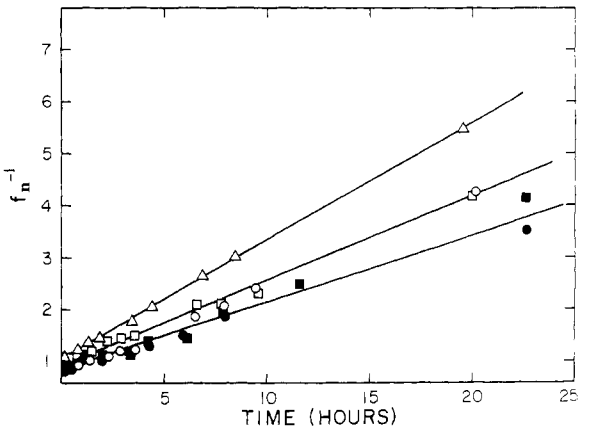

FIGURE 3: The effect of initial protein concentration on the rate of the near- and far-uv CD spectral changes of Dip-subtilisin Novo in Gdn·HCl. The protein samples contained 5.23 *M* Gdn·HCl and 0.08 *M* sodium acetate buffer at pH 6.1. f_n was calculated from the near- (278 nm) and far- (222 nm) uv CD. The following protein concentrations were employed: 0.06 mg/ml, 278 nm (●); 0.06 mg/ml, 222 nm (■); 0.25 mg/ml, 278 nm (○); 0.25 mg/ml, 222 nm (□); 2.2 mg/ml, 278 nm (△).

Ultra Pure grade (Heico Co., Delaware Water Gap, Pa.). All other chemicals were reagent grade.

The diisopropylphosphoryl (Dip) derivatives of subtilisins Novo and Carlsberg were prepared by the procedure of Markland (1969) and found to be devoid of detectable activity when assayed using *N-trans*-cinnamoylimidazole (Bender et al., 1966). Protein stock solutions free of autolyzed material were prepared by gel filtration using short (2 × 30 cm) columns of Sephadex G-75. Protein fractions were pooled and concentrated by ultrafiltration through Diaflow UM-10 ultrafilters (Amicon Corp., Lexington, Mass.). Concentrations were determined spectrophotometrically at 278 nm using $E_{1cm}^{1\%}$ = 8.6 for Dip-subtilisin Carlsberg (Guntelberg and Otteson, 1954) and $E_{1cm}^{1\%}$ = 11.7 for Dip-subtilisin Novo (Matsubara et al., 1965; Markland and Smith, 1971). The protein stock solutions were monitored periodically for autolysis by gel filtration. Appreciable amounts of dephosphonylation and subsequent autolysis were observed in protein stock solutions held at room temperature, but these processes were undetectable in protein solutions (7–10 mg/ml) stored at 4° for several weeks or more.

Circular Dichroism Studies. CD spectra were obtained using a Durrum-Jasco J-20 recording spectropolarimeter. Ellipticity values are reported in the units deg cm^2 dmol^{-1}. The mean residue weight for subtilisins was taken as 100. The instrument was calibrated at 290 nm with camphorsulfonic acid-d_{10} (DeTar, 1969) and with hen egg white lysozyme at 208 and 222 nm (Chen et al., 1972). Unless otherwise noted the spectra represent the average of at least two scans.

Gel Filtration Studies. Gel filtration analysis of protein solutions which had been incubated in concentrated solutions of Gdn·HCl was performed using 2 × 30 cm columns of Sephadex G-75 employing either 0.1 *M* NaCl or KCl at a nominal pH of 7 as the eluent.

Results

Effect of Gdn·HCl on the CD of Subtilisin Enzymes. Near- and far-uv CD spectra of Dip-subtilisin Novo and Dip-subtilisin Carlsberg exhibit slow and irreversible changes with time in concentrated solutions of Gdn·HCl.

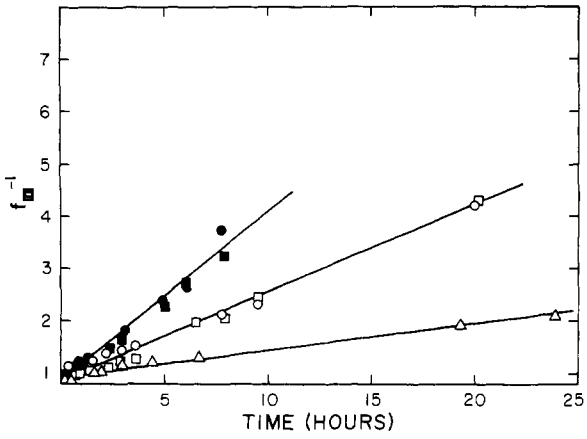

FIGURE 4: The effect of pH on the rate of the spectral changes in the near- and far-uv CD of Dip-subtilisin Novo. The samples contained 0.25 mg/ml of protein, 0.08 *M* sodium acetate buffer, and 5.23 *M* Gdn·HCl. f_n was calculated from the near- (278 nm) and far- (222 nm) uv CD. The following pH values were employed: pH 7.2, 278 nm (△); pH 6.0, 278 nm (○); pH 6.0, 222 nm (□); pH 5.6, 278 nm (●); pH 5.6, 222 nm (■).

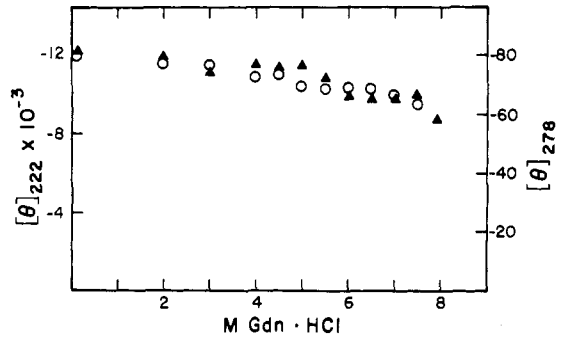

FIGURE 5: Molar ellipticity of Dip-subtilisin Carlsberg as a function of Gdn·HCl concentration ca. 30 min after solution. The spectra were recorded in the near-uv at 278 nm (○) and the far-uv at 222 nm (▲). The solutions used for the near-uv CD measurements contained 1.4 mg/ml of protein and 0.06 *M* sodium acetate buffer at pH 5.9. The solutions used for the far-uv CD measurements contained 0.29 mg/ml of protein and 0.06 *M* sodium acetate buffer at pH 5.9.

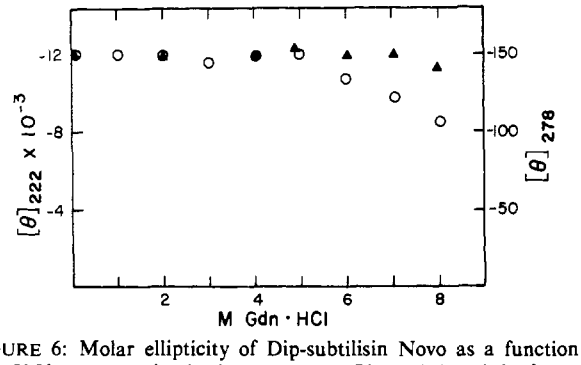

FIGURE 6: Molar ellipticity of Dip-subtilisin Novo as a function of Gdn·HCl concentration in the near-uv at 278 nm (○) and the far-uv at 222 nm (▲). The spectra were recorded ca. 30 min after solution. The solutions used for the near-uv CD measurements contained 1.4 mg/ml of protein and 0.08 *M* sodium acetate buffer at pH 6.1. The far-uv solutions contained 0.17 mg/ml of protein and 0.08 *M* sodium acetate buffer at pH 6.1.

Examples of such temporal changes are shown for Dip-subtilisin Novo in 7.0 *M* Gdn·HCl in Figure 1. In both spectral regions a gradual decrease in ellipticity with time is noted at high Gdn·HCl concentrations. The 278–279 nm extremum in the near-uv CD sustains a slight (2–3 nm) red shift while the far-uv CD component at 222 nm is gradually replaced by the tail of a new spectral component displaced to lower wavelength. The extreme opacity of Gdn·HCl in the far-uv prevents a determination of the complete spectrum of this component. Little change, if any, in the *initial* position of the near- and far-uv CD bands with increasing Gdn·HCl concentration is apparent.

Attempts were made to reverse the slow spectral changes by a variety of methods, including dialysis, ultrafiltration, and dilution of the denaturant additive. All of these experiments were uniformly unsuccessful, indicating that the observed spectral changes are irreversible.

The nature of the slow spectral changes was further investigated for both inactivated subtilisins Novo and Carlsberg by recording CD spectra as a function of time in the presence of different concentrations of Gdn·HCl, different initial protein concentrations, and at various pH values. In both the near- and far-uv spectral regions the data were describable within experimental error by second-order kinetics. First-order kinetic plots were invariably curved. The kinetic data are expressed as a plot of f_n^{-1} vs. time where f_n is the fraction of molecules in the initial or "native" state calculated from the following equation:

$$f_n = (\theta_t - \theta_\infty)/(\theta_0 - \theta_\infty) \quad (1)$$

where θ_t is the ellipticity at time t, measured at either 222 or 278 nm, and θ_0 and θ_∞ are the ellipticities at zero time and infinite time, respectively.

Figure 2 illustrates the time dependence of the slow CD changes evident in both the near- and far-uv CD spectral regions of Dip-subtilisin Novo with increasing concentrations of denaturant at a fixed initial concentration of protein. The reaction rate depends on the concentration of Gdn·HCl with higher denaturant concentrations effecting a more rapid destabilization of the molecule. The rates of change in both the near- and far-uv spectral regions are identical. At a denaturant concentration below 4 *M* the time course of the CD changes is very slow, and in the absence of Gdn·HCl the inactivated protein appears to be stable and resistant to autolysis for at least a day at room temperature.

In the presence of a fixed amount of denaturant the rate of the CD changes depends on protein concentration with higher concentrations effecting a greater reaction rate. In Figure 3 is shown the effect of increasing protein concentration on the rate of the CD changes observed for Dip-subtilisin Novo in 5.2 *M* Gdn·HCl. Decreasing the solution pH while maintaining fixed protein and denaturant concentrations results in a similar increase in the rate of the CD changes. This is illustrated in Figure 4 for Dip-subtilisin Novo in 5.2 *M* Gdn·HCl over the pH range 5.6–7.2. As noted earlier, identical rates of change are observed in both the near- and far-uv spectral regions.

Because of the irreversible time-dependent CD changes it is not possible to obtain equilibrium spectra for subtilisin enzymes under denaturing conditions such as created by high concentrations of Gdn·HCl. Since the time course of the CD spectral changes is sufficiently slow it is reasonable to consider spectra recorded within 30 min of solution as *approximations* of the true equilibrium spectra which would be obtained in the absence of irreversible changes. Figures 5 and 6 depict the transition curves obtained by monitoring the far- (222nm) and near- (278 nm) uv CD of Dip-subtilisins Novo and Carlsberg as a function of Gdn·HCl concentration. The remarkable stability of the native structures of both subtilisin enzymes to concentrations of

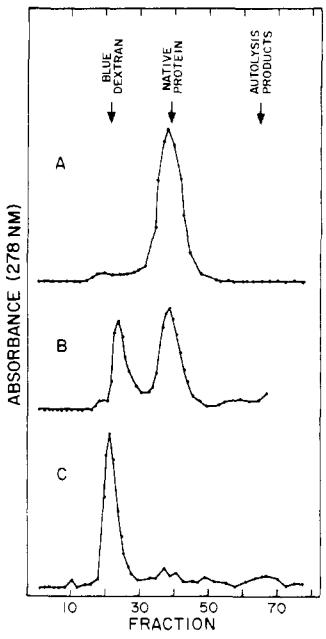

FIGURE 7: Time course of elution profile changes of Dip-subtilisin Novo in Gdn·HCl. Profiles A, B, and C were obtained by chromatographing a 1.4 mg/ml protein sample after 1, 3.2, and 24 hr incubation in 7.13 *M* Gdn·HCl at pH ca. 7. The elution volumes of various materials are indicated by arrows.

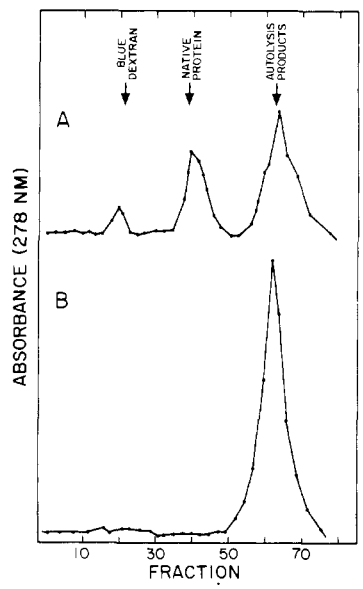

FIGURE 8: Time course of elution profile changes of uninhibited subtilisin Novo in Gdn·HCl. Profiles A and B were obtained by chromatographing a 2.5 mg/ml protein sample after 13 min and 3.5 hr incubation in 5.23 *M* Gdn·HCl at pH ca. 6. The elution volumes of various materials are indicated by arrows.

Gdn·HCl as high as 8 *M* is evident in both the near- and far-uv spectral regions. The breaks in the transition curves at high denaturant concentrations are attributable to irreversible changes which occurred during the time between solution of the protein sample and recording the spectra.

Gel Filtration Behavior of Subtilisin Enzymes in Gdn·HCl Solutions. The time-dependent CD changes exhibited by subtilisin enzymes in concentrated solutions of Gdn·HCl were further characterized by gel filtration studies. The elution profiles of Dip-subtilisin Novo after varying times of exposure to 7.1 *M* Gdn·HCl at 22–23° are depicted in Figures 7A–C. As the time-dependent changes proceed to completion the native protein fraction is replaced by a high molecular weight fraction which appears at the void volume of the Sephadex G-75 column effluents. After 1 hr ca. 90% of the original protein fraction is present, with the remainder appearing earlier in the column effluent. At 3 hr the intact protein fraction is still apparent but about 50% of the total protein appears in the high molecular weight fraction. After 6 hr the percentage of intact protein decreases to ca. 40–45%, and after 24 hr exposure to Gdn·HCl little intact protein remains with ca. 90% or greater of the original protein material appearing at the void volume. No low molecular weight autolysis products were detected in this experiment. The time course of these chromatographic changes parallels closely the slow irreversible CD changes observed in the same solutions of Dip-subtilisins Novo or Carlsberg.

The appearance of a high molecular weight fraction with time is indicative of an intermolecular association of protein molecules; however, the possibility exists that unfolded protein is autolyzed by residual activity remaining in the preparation, and that it is the low molecular weight autolysis products which associate in the presence of high Gdn·HCl concentrations. To examine this possibility the identical experiment was performed with solutions of subtilisins Novo and Carlsberg in Gdn·HCl which were not inactivated with Dip-F. The changes observed in the CD of solutions of subtilisin Novo in Gdn·HCl were found to be much faster than the corresponding solutions of Dip-subtilisin Novo, being complete in 1–2 hr. The elution profiles of aliquots of subtilisin Novo chromatographed after 13 min and 3.5 hr in 5.2 *M* Gdn·HCl are shown in Figures 8A and B. Again the time course of the gel filtration changes parallels that of the CD changes; however, it is noted that the final product is instead a low molecular weight fraction ascribable to autolysis. Three peaks are apparent in the elution profile after 13 min, a peak eluting with a partition coefficient characteristic of the intact protein, and peaks corresponding to *both* associated and autolyzed material. Similar results were obtained for active subtilisin Carlsberg. Apparently aggregates are also formed in solutions of the uninhibited enzyme initially, but these are rapidly autolyzed along with the native protein to yield low molecular weight polypeptide fragments which do not associate in Gdn·HCl.

Effect of Other Denaturants. The effect of other denaturants on the CD of Dip-subtilisin Novo was examined and is summarized below. In all cases the spectral changes produced, if any, were similar to those produced by Gdn·HCl. Extremes of pH (below 4 and above 12) produce irreversible CD changes which gel filtration experiments demonstrate to be accompanied by aggregation. The use of temperature as a denaturant is complicated by increased rates of dephosphonylation and autolysis which occur at elevated temperatures. Irreversible changes occur in the CD of Dip-subtilisin Novo above 60° at pH 7.8 and above 50° at pH 4.2. Dioxane (40%) and 4 *M* $NaClO_4$ were found to have no effect on the CD of Dip-subtilisin Novo. The effect of Gdn·SCN[1] appears similar to that of Gdn·HCl although the state of protein association in this solvent was not investigated.

Discussion

In contrast to many other globular proteins subtilisin enzymes exhibit an extraordinary conformational stability in the presence of denaturants. The inactivated derivatives of these proteins at neutral pH are able to withstand Gdn·HCl

concentrations as high as 4 *M* with little consequence. The work described in this paper shows that at concentrations above 4 *M* irreversible conformational changes occur slowly with time which are paralleled by the appearance of aggregated protein. These time-dependent changes are slow in comparison with the first-order kinetic events observed for the reversible unfolding of less stable globular proteins (Barnard, 1964; Tanford et al., 1966; Ikai and Tanford, 1971). The near-uv CD at 278–279 nm is sensitive to details of protein secondary and tertiary structure in the vicinity of "buried" aromatic residues (M. F. Brown and T. Schleich, manuscript in preparation) and exhibits a loss of optical activity with time suggesting an unfolding of the molecule. The parallel changes which occur in the far-uv are interpreted as a transition from the native form which contains appreciable contributions from α-helical secondary structure (Greenfield and Fasman, 1969; Wright et al., 1969) to a denatured species exhibiting a single CD minimum obscured by the solvent additive at wavelengths below 210 nm, similar to that exhibited by randomly coiled polypeptide chains (Greenfield and Fasman, 1969; Timasheff, 1971). Furthermore, the time course of the changes in both spectral regions is identical suggesting that the optical activity at these wavelengths is sensitive to the same process, and hence the conformational change which occurs must involve most or all of the protein molecule. Markland (1969) has observed similar time-dependent changes in the uv absorption difference spectra of subtilisin enzymes in 5 *M* Gdn·HCl.

Evidence for aggregation of subtilisin enzymes in the presence of Gdn·HCl is provided by the nature of the spectral changes and by gel filtration studies. A 2–3-nm red shift is sustained in the far-uv with time which is interpreted as a stabilization of the optically active π–π^* aromatic transitions by an increase in local polarizability upon association (McRae, 1957; Bigelow and Geschwind, 1960; Yanari and Bovey, 1960). The CD changes with time are paralleled by the appearance of increasing amounts of high molecular weight material emerging at the column void volume. Aggregates are also formed initially in solutions of the uninhibited enzyme in Gdn·HCl but these are rapidly autolyzed to yield low molecular weight polypeptide fragments which do not associate. While seemingly identical CD spectral changes are produced by autolysis and upon aggregation of unfolded protein molecules, these processes are readily distinguished by gel filtration.

The autolysis of subtilisin enzymes which have not been inactivated is apparently due to the low specificity of these enzymes and the increased susceptibility of unfolded molecules produced by denaturants to proteolytic digestion by the remaining population of active enzyme. Gel filtration studies of partially autolyzed subtilisin preparations in the presence or absence of denaturants reveal only two fractions of symmetrical peak shape corresponding to native intact protein and the low molecular weight products of autolysis. Since partially autolyzed or "nicked" protein material is not resolved in the gel filtration experiments it is unlikely that the irreversibility of the CD changes is a consequence of limited peptide hydrolysis, which might prevent reattainment of the native configuration upon removal of denaturant. Furthermore, end group analysis of subtilisin enzymes which are inactivated by Dip-F reveals a single N terminus (Otteson and Schellman, 1957).

Our studies suggest that inactivated enzyme preparations must be employed for denaturation studies of proteolytic enzymes of low specificity such as the subtilisins if autolysis is to be avoided. Several studies of active subtilisin preparations in the presence of denaturants have led to contradictory interpretations. For example, Herskovits and Fuchs (1972) concluded that urea denaturation of active subtilisin Novo leads to full exposure of the tryptophanyl and tyrosyl residues of this enzyme. A similar study by Bernhard et al. (1965) reported the urea denaturation of subtilisin Novo which had been acylated using a series of acylimidazole compounds. Gounaris and Otteson (1965), on the other hand, have stated that subtilisin Novo is stable and active in 6 *M* urea. The different interpretations may be explained by the existence of complete or limited autolysis under the conditions employed by Herskovits and Fuchs (1972) and Bernhard et al. (1965). Stauffer and Sullivan (1971) reported that the viscosity and optical rotatory dispersion of active subtilisin Carlsberg are unchanged in 10 *M* urea or 6 *M* Gdn·HCl, suggesting that this enzyme is not unfolded or autolyzed in concentrated denaturant solutions. This would appear to be at odds with our gel filtration experiments of active subtilisin preparations which reveal considerable autolysis within 10 min of solution in Gdn·HCl. The uv difference spectral experiments of Markland (1969) suggest that all the abnormally titrating tyrosyl residues of Dip-subtilisin Carlsberg are normalized in 5 *M* Gdn·HCl, which is inconsistent with the results of Stauffer and Sullivan (1971). The time-dependent denaturation and aggregation of inactive subtilisin enzymes may account for the normalization of tyrosyl residues since Markland's experiments were not commenced until ca. 3 or more hr incubation of the enzyme in concentrated Gdn·HCl solutions.

The slow irreversible changes in the CD of Dip-subtilisin enzymes are describable within experimental error by second-order kinetics, viz., by plots of f_n^{-1} vs. time which are observed to be linear. Studies of the time dependence of reversible folding and unfolding transitions of other proteins in concentrated Gdn·HCl solutions have revealed an adherence to first-order kinetics (Barnard, 1964; Tanford et al., 1966; Ikai and Tanford, 1971; Henkins and Turner, 1973; McKenzie and Ralston, 1973). Ralston (1974) has recently examined the irreversible unfolding of $PhCH_2SO_2$-subtilisin Novo induced by relatively low (2–4 *M*) concentrations of Gdn·HCl. Under the acidic conditions employed by Ralston (1974) the protein is more susceptible to Gdn·HCl induced denaturation than at the near neutral pH values employed in this study, and follows first-order kinetics for the first 30 min. At lower denaturant concentrations above pH 5 slow reactions exhibiting anomalous kinetics were observed by Ralston (1974). These reactions are in the time domain of our study and could correspond to the slow second-order kinetic events described in this paper.

The mechanism of Gdn·HCl induced protein denaturation is still not thoroughly understood. It is generally agreed that these denaturants destabilize biopolymer conformations in part by disrupting both hydrogen bonds and hydrophobic bonds, but an assessment of the relative importance of each of these contributions appears to be equivocal (Kauzmann, 1959; Schick, 1964; Wetlaufer et al., 1964; Robinson and Jencks, 1965a,b; Neméthy, 1967). Tiffany and Krimm (1973) have recently argued that Gdn·HCl acts primarily through the disruption of hydrogen bonding involving the polypeptide backbone carbonyl groups, thereby destabilizing secondary and tertiary protein structures. The high ionic strength of concentrated Gdn·HCl solutions precludes significant contributions from ionic interactions such

as salt bridges or bound ions and disulfide bonds are absent in these enzymes. Thus the unusual stability of subtilisin enzymes must reside in hydrogen bonding or hydrophobic bonding interactions, as is the case with less stable enzymes. The subtilisin enzymes exhibit the slowest amide proton exchange rates of any protein studied to date (Johansen and Ottesen, 1974), suggesting "tighter" (i.e., less conformational breathing) secondary and tertiary structures than those of less stable enzymes. The exceptional conformational stability of subtilisins may be a consequence of structural rigidity, thereby denying access of denaturants to the hydrogen-bonded protein interior. If the principal mode of action of Gdn·HCl is to disrupt hydrogen bonding then the observed insolubility and association of denatured protein molecules in Gdn·HCl can be rationalized in terms of unfolding of the molecule, followed by association about exposed nonpolar sites incapable of complete solvation.

Association of proteins in aqueous solutions of Gdn·HCl is not without precedent. Thermally denatured chymotrypsinogen is insoluble at less than 3 *M* Gdn·HCl (Aune et al., 1967), hen egg-white lysozyme displays some association after several hours in solution (Tanford et al., 1966), and spin-labeled chymotrypsin aggregates severely in Gdn·HCl (Morrisett and Broomfield, 1971). The exceptional stability of subtilisin enzymes to Gdn·HCl and the very high denaturant concentrations necessary for unfolding seem to produce effects either nonexistent or only casually noticed in less stable proteins.

References

Aune, K. C., Salahuddin, A., Zarlengo, M. H., and Tanford, C. (1967), *J. Biol. Chem. 242,* 4486.

Barnard, E. A. (1964), *J. Mol. Biol. 10,* 235.

Bender, M. L., Begué-Canton, M. L., Blakeley, R. L., Brubacher, L. J., Feder, J., Gunther, C. R., Kézdy, F. J., Killheffer, J. V., Jr., Marshall, T. H., Miller, C. G., Roeske, R. W., and Stoops, J. K. (1966), *J. Am. Chem. Soc. 88,* 5890.

Bernhard, S. A., Lau, S. J., and Noller, H. (1965), *Biochemistry 4,* 1108.

Bigelow, C. C., and Geschwind, I. I. (1960), *C. R. Trav. Lab. Carlsberg 31,* 283.

Chen, Y. H., Yang, J. T., and Martinez, H. M. (1972), *Biochemistry 11,* 4120.

DeTar, D. F. (1969), *Anal. Chem. 41,* 1406.

Drenth, J., Hol, W. G. J., Jansonius, J. N., and Koekoek, R. (1972), *Cold Spring Harbor Symp. Quant. Biol. 36,* 107.

Gounaris, A., and Otteson, M. (1965), *C. R. Trav. Lab. Carlsberg 35,* 37.

Greenfield, N., and Fasman, G. D. (1969), *Biochemistry 8,* 4108.

Guntelberg, A. V., and Otteson, M. (1954), *C. R. Trav. Lab. Carlsberg, Ser. Chim. 29,* 36.

Henkins, R. W., and Turner, S. R. (1973), *Biochemistry 12,* 1618.

Herskovits, T. T., and Fuchs, H. H. (1972), *Biochim. Biophys. Acta 263,* 468.

Ikai, A., and Tanford, C. (1971), *Nature (London) 230,* 100.

Johansen, J. T., and Ottesen, M. (1974), *Isr. J. Chem. 12,* 339.

Kauzmann, W. (1959), *Adv. Protein Chem. 14,* 1.

Markland, F. S. (1969), *J. Biol. Chem. 244,* 694.

Markland, F. S., and Smith, E. L., (1971), *Enzymes, 3rd Ed., 3,* 561.

Matsubara, H., Kasper, C. B., Brown, D. M., and Smith, E. L. (1965), *J. Biol. Chem. 240,* 1125.

McKenzie, H. A., and Ralston, G. B. (1973), *Biochemistry 12,* 1025.

McRae, E. G. (1957), *J. Chem. Phys. 61,* 562.

Morawetz, H. (1972), *Adv. Protein Chem. 26,* 243.

Morrisett, J. D., and Broomfield, C. A. (1971), *J. Am. Chem. Soc. 93,* 7297.

Neméthy, G. (1967), *Angew. Chem. 6,* 195.

Olaitan, S. A., DeLange, R. J., and Smith, E. L., (1968), *J. Biol. Chem. 243,* 5296.

Otteson, M., and Schellmam, C. G. (1957), *C. R. Trav. Lab. Carlsberg, Ser. Chim. 30,* 157.

Ralston, G. B. (1974), *C. R. Trav. Lab. Carlsberg 39,* 399.

Robertus, J. D., Alden, R. A., and Kraut, J. (1971), *Biochem. Biophys. Res. Commun. 42,* 334.

Robinson, D. R., and Jencks, W. P. (1965a), *J. Am. Chem. Soc. 87,* 2462.

Robinson, D. R., and Jencks, W. P. (1965b), *J. Am. Chem. Soc. 87,* 2470.

Schick, M. J. (1964), *J. Phys. Chem. 68,* 3585.

Stauffer, C. E., and Sullivan, J. F. (1971), *Biochim. Biophys. Acta 251,* 407.

Stauffer, C. E., and Treptow, R. W. (1973), *Biochim. Biophys. Acta 295,* 457.

Tanford, C., Pain, R. H., and Otchin, N. S. (1966), *J. Mol. Biol. 15,* 489.

Tanford, C. (1968), *Adv. Protein Chem. 23,* 121.

Tiffany, M. L., and Krimm, S. (1973), *Biopolymers 12,* 575.

Timasheff, S. N. (1971), *Enzymes, 3rd Ed., 2,* 371.

Tsuru, D. (1969), *Kagaku To Kogyo (Osaka), 43,* 199 (cited in *Chem. Abstr. 71,* 79183*j*).

Wetlaufer, D. B., Malik, S. K., Stoller, L., and Coffin, R. L. (1964), *J. Am. Chem. Soc. 86,* 508.

Wright, C. S., Alden, R. A., and Kraut, J. (1969), *Nature (London) 221,* 235.

Yanari, S., and Bovey, F. A. (1960), *J. Biol. Chem. 235,* 2518.