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Effect of Long-Chain Alkyl Sulfate Binding on Circular Dichroism and Conformation of Soybean Trypsin Inhibitor†

Eigo Mori and Bruno Jirgensons*

ABSTRACT: The disorganization and helix formation process of "Kunitz" soybean trypsin inhibitor (STI) effected by sodium dodecyl sulfate binding was investigated by the circular dichroism (CD) probe. The binding isotherms of dodecyl sulfate to STI were determined at the ionic strength of 0.033, 0.12, and 0.25 at pH 7.3, 25 °C. The perturbation and disorganization of this nonhelical protein were observed at an early binding stage ($\bar{\nu}$, the average molar ratio of bound detergent to STI, up to about 7 in the case of the isotherm at $I = 0.12$). The disappearance of a positive CD peak at 226 nm and appearance of a negative CD band at 239 nm took place at this step and were affected by the number of carbon atoms in the alkyl group of detergents. The transition of the polypeptide backbone into a more ordered conformation proceeded gradually during cooperative binding of dodecyl sulfate molecules. An abrupt increase of detergent binding occurred near the critical micelle concentration of the detergent. The helix formation was completed prior to this step ($\bar{\nu} = 30$, at $I = 0.12$).

Many studies have been carried out with the aim of understanding interactions between protein and detergent, especially sodium dodecyl sulfate ($NaDodSO_4$),[1] for four decades. Despite accumulation of a wealth of knowledge on this subject [for reviews, see Tanford (1968), Steinhardt & Reynolds (1969), and Lapanje (1978)], the exact nature of protein–dodecyl sulfate complex has not been conclusively elucidated. The increase in the helix content of some proteins upon complexing with sodium dodecyl sulfate has been observed by optical activity measurements (Meyer & Kauzmann, 1962; Jirgensons, 1966; Reynolds & Tanford, 1970a; Visser & Blout, 1971). Recently, conformational properties of the complexes of 15 proteins were analyzed by circular dichroism. The complexes were shown to be a mixture of random coil and α helix (Mattice et al., 1976). However, not much attention has been paid to the process of helix formation induced by $NaDodSO_4$ binding.

Soybean trypsin inhibitor (Kunitz) is a well-characterized stable protein [for review, see Laskowski & Sealock (1971)].

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[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; STI, soybean trypsin inhibitor (Kunitz); CD, circular dichroism; UV, ultraviolet; I, ionic strength; $\bar{\nu}$, average molal ratio of bound $NaDodSO_4$ to STI. The values reported in this paper were calculated by using the binding isotherm at $I = 0.12$ (Figure 1).

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The protein has little regular structure with no helix and only a negligible amount of the β-pleated-sheet structure as revealed by X-ray crystallography (Sweet et al., 1974). The circular dichroic analysis of soybean trypsin inhibitor (STI)–sodium dodecyl sulfate ($NaDodSO_4$) complexes at higher $NaDodSO_4$ concentrations has been reported from this laboratory (Jirgensons & Capetillo, 1970; Jirgensons & Shimazaki, 1975). In view of the structural feature mentioned above, STI seems to be a suitable protein for detailed examination of the action of $NaDodSO_4$ as a helix-promoting agent and the helix formation process of a nonhelical protein. Of the earlier studies on the conformaton of STI, the work of Wu & Scheraga (1962), Steiner & Edelhoch (1963), and Baba et al. (1969) can be mentioned.

The present communication deals with a further examination of denaturation and reorganization of STI effected by $NaDodSO_4$ binding. The whole process observed so far could be separated into several consecutive transitions and characterized more definitely by several different measurements.

Materials and Methods

Materials. Soybean trypsin inhibitor (Kunitz) was an electrophoretically homogeneous preparation (lot SI 55N505X) obtained from Worthington Biochemical Corp. Sodium dodecyl sulfate used was a most purified product supplied by British Drug Houses (lot 2200980). Other alkyl sulfates were high purity materials purchased from Schwarz/Mann. *p*-(Dimethylamino)azobenzene, obtained from Aldrich Chemical Co., was recrystallized 3 times from acetone–water mixtures. All other chemicals were of analytical grade.

Methods. Binding isotherms were determined by equilibrium dialysis of 1.0 mL of buffered protein solutions against 10 mL of $NaDodSO_4$ solutions containing no protein at I = 0.25, 0.12, and 0.033. Visking dialysis tubings were acetylated and washed as described by Pitt-Rivers & Impiombato (1968). The protein was placed in a bag of Visking tubing at a concentration of 0.2% in phosphate buffer, pH 7.3. Dialysis was carried out against a given concentration of $NaDodSO_4$ in the same buffer containing 0.02% sodium azide with continuous stirring by a magnetic stirrer at 25 °C. The dialysis was continued for 5–18 days (5–7 days in a low concentration of the detergent and 14–18 days in a high $NaDodSO_4$ concentration near the critical micelle concentration). For attainment of equilibrium, duplicated dialysis was performed, and the period of dialysis was changed to determine each point on the isotherm. The results from different dialysis times were found identical. After the completion of dialysis, the $NaDodSO_4$ concentration of the outside solution was analyzed by the method of methylene blue–$NaDodSO_4$ complex formation (Mukerjee, 1956). Actual procedures followed the method improved by Hayashi (1975). The amount of $NaDodSO_4$ bound to STI was calculated from the total amount of $NaDodSO_4$, the amount of free $NaDodSO_4$ (determined from the analysis of the $NaDodSO_4$ concentration in the outside solution), and the amount of protein used. All the data were corrected by $NaDodSO_4$ blank dialysis (STI omitted) done at the same time. The circular dichroic (CD) spectrum of dialysates was also checked.

The CD spectrum was recorded at an ambient room temperature (23 ± 2 °C) with a Durrum-Jasco CD-SP instrument improved by D. Sproul of Sproul Scientific Instruments. In kinetical studies, a water-jacketed cell was used to keep the sample solution at 25 °C. In most CD recordings, the sensitivity scale setting was 5×10^{-5} dichroic absorption per 1 cm on the recorder chart. A cell with 1.0-cm light path was used in the near-UV range, and a cell with 0.1-cm light path was used in the far-UV range measurement. The concentration of STI was, unless otherwise specified, 0.10% in the near-UV region and 0.01% in the far-UV region. In most cases, two protein samples were used to determine a CD spectrum. The CD spectra are expressed in mean residue ellipticities $[\theta]$, deg·cm^2·dmol^{-1}, taking the value of 110 for the mean residue weight. The ellipticity curves were calculated from duplicate recording. The range of the experimental errors in calculation of $[\theta]$ values was $\pm 0.7 \times 10^3$ deg·cm^2·dmol^{-1} around 200 nm, $\pm 0.1 \times 10^3$ deg·cm^2·dmol^{-1} around 220 nm, and ±1.4 deg·cm^2·dmol^{-1} around 275 nm. The instrument was calibrated with *d*-10-camphorsulfonic acid (Chen & Yang, 1977).

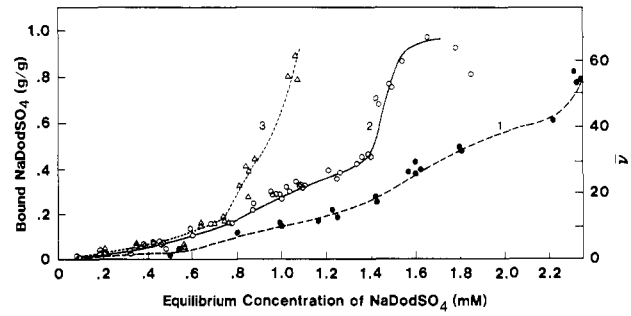

FIGURE 1: Binding isotherms of $NaDodSO_4$ to STI at pH 7.3 (25 °C). Curves 1, 2, and 3 represent I = 0.033, 0.12, and 0.25, respectively.

Dye solubilization with (dimethylamino)azobenzene was performed in pH 7.3 phosphate buffer of I = 0.12 at 25 °C by the following procedures modified from the method of Steinhardt et al. (1974). Approximately 5 mg of the dye crystals was added to the $NaDodSO_4$ solutions of various concentrations (from 0.29 to 5.8 mg) containing 0.1% STI in a total volume of 4.0 mL in test tubes. The solutions were stirred by a magnetic stirrer for 48 h, and the absorbance of supernatants was measured at 415 nm after centrifugation at 3000 rpm for 10 min. The blank measurements in the absence of STI or $NaDodSO_4$ were also done in the same run. The critical micelle concentration of $NaDodSO_4$ was found to be 1.6×10^{-3} M (I = 0.12) at pH 7.3 (25 °C) by measurements of dye solubilization (in Figure 5).

Protein concentrations were calculated by using the extinction coefficient $E_{1\%}^{1cm}$ = 10.13 at 280 nm for STI (Yamamoto & Ikenaka, 1967). The protein concentration determination and analyses of $NaDodSO_4$ and (dimethylamino)-azobenzene concentrations were carried out on a Beckman DU spectrophotometer.

Results

Binding Isotherms. The binding isotherms of $NaDodSO_4$ to intact STI[2] at I = 0.25, 0.12, and 0.033 (pH 7.3, 25 °C) are shown in Figure 1. Three curves clearly separate from each other at about 0.6 mM (equilibrium $NaDodSO_4$ concentration). All isotherms showed a steep rise at about 0.75 (I = 0.25), 1.4 (I = 0.12), and 2.3 mM (I = 0.033) after a

[2] Some of the binding isotherms of detergents including $NaDodSO_4$ reported so far are for protein polypeptides which are obtained from proteins by reductive cleavage of all disulfide bridges and denaturation with guanidine hydrochloride (Reynolds & Tanford, 1970b; Takagi et al., 1975; Makino & Niki, 1977). Since the CD spectra of STI in the near-UV region are altered upon partial reduction by dithioerythritol (Tamura et al., unpublished experiments), we determined the isotherms to initially native STI instead of STI polypeptide in consideration of the purpose of this study.

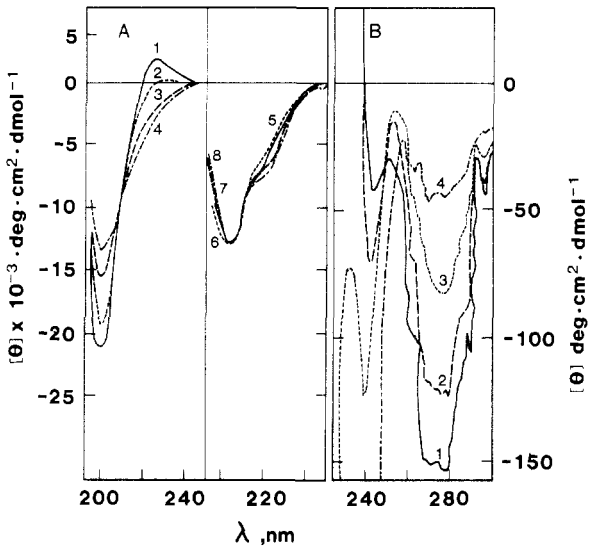

FIGURE 2: CD spectra of STI–$NaDodSO_4$ complex: $I = 0.12$; phosphate buffer, pH 7.3. (A) (1) native STI, $\bar{\nu} = 0$; (2) $\bar{\nu} = 5$; (3) $\bar{\nu} = 11$; (4) $\bar{\nu} = 19$; (5) (---) $\bar{\nu} = 25$; (6) (—) $\bar{\nu} = 40$; (7) (– –) $\bar{\nu} = 65$; (8) (·-·) $\bar{\nu} = 65$ (reduced STI). Protein concentration, 0.01%. (B) (1) native STI, $\bar{\nu} = 0$; (2) $\bar{\nu} = 2$; (3) $\bar{\nu} = 5$; (4) $\bar{\nu} = 25$. Protein concentration, 0.1%.

slow increase of detergent binding. We used the isotherm at $I = 0.12$ as a standard binding curve for further experiments only on the grounds of experimental convenience.

The binding constants of dodecyl sulfate anion for STI were calculated from the binding isotherm at $I = 0.12$ by a reciprocal plot (Reynolds et al., 1967). At low $\bar{\nu}$ (up to 7), the reciprocal plot was linear, the estimated association constant, K, was 0.8×10^3, and the number of binding sites, n, was 18. The value of K is approximately 1000 times smaller than that for bovine serum albumin (Reynolds et al., 1967). This indicates that STI has a much lower affinity than albumin to dodecyl sulfate anion. Above $\bar{\nu} = 7$, the plot deviated from the straight line, suggesting that a cooperative process due to a conformational change of the protein occurred.

The maximal amount of $NaDodSO_4$ bound increased by reduction of two disulfide bonds, 0.93 g/g of STI for initially native STI and 1.0 g/g of STI for reduced STI. The presence of disulfide cross-linkage tends to diminish $NaDodSO_4$ binding, as was reported for other proteins (Pitt-Rivers & Impiombato, 1968).

CD Spectra of STI–$NaDodSO_4$ Complexes. Figure 2 shows the CD spectra of STI–$NaDodSO_4$ complexes as a function of the detergent binding ($\bar{\nu}$). At $\bar{\nu} = 2$–3 (Figure 2, curve B-2), the spectrum of the complex is still similar to that of native STI (curve B-1), but the intensity of the 226-nm peak originating partly from tyrosine (Baba et al., 1969; Jirgensons & Shimazaki, 1975) and the 290–270-nm zone considerably decreased. The negative bands at 287 and 294 nm (due to tyrosyl and tryptophanyl chromophores) were also perturbed.

A new negative extremum was observed at 239 nm at $\bar{\nu} = 5$ (Figure 2, curve B-3) just after the large positive peak at 226 nm was almost abolished. This negative band soon disappeared (at $\bar{\nu} = 6$) and has a transient character. The CD spectra of the complex at $\bar{\nu} = 5$ (Figure 2, curves A-2 and B-3) resemble those obtained in the presence of alcohol (for example, 2.61 M 1-propanol) in both UV regions (Tamura & Jirgensons, 1980). In terms of effectiveness of inducing the CD band, $NaDodSO_4$ is a much more potent perturbant than alcohol.

The changes in the near-UV region completed below $\bar{\nu} \sim 10$ and remained almost identical in the following steps, while the conformational change of the main polypeptide chain appears to occur largely above $\bar{\nu} \sim 10$. The amplitude of the negative peak at 200 nm decreased between $\bar{\nu} = 5$ and $\bar{\nu} = 19$, and the position of the band was shifted from 200 to 205 nm above $\bar{\nu} = 20$. Although it is quite difficult to determine unequivocally when the transformation into a periodic structure is initiated during the $NaDodSO_4$ binding to STI, the spectrum (Figure 2, curve A-5) has a shoulder around 220 nm, suggesting that helical structure is gradually formed as more $NaDodSO_4$ molecules bind to STI. In the spectra (Figure 2, curves A-6 and A-7), the presence of helical structure is evidenced by the intensified shoulder. The helix content in the completely transitioned STI–$NaDodSO_4$ complexes (Figure 2, curves A-7 and A-8) was estimated to be 22% from the band centered at 220 nm, taking the value of −30 000 for the helix as the standard (Chen et al., 1974). The helix formation was promoted slightly in the case of the reduced STI–$NaDodSO_4$ complex (Figure 2, curve A-8), suggesting that the disulfide bridges do not hamper the folding into helical conformation, as was shown with the effect of 2-chloroethanol (Ikeda et al., 1968).

Kinetics. The changes in the CD spectra of STI caused by $NaDodSO_4$ binding, which are represented in Figure 2, occur fairly slowly. Rate studies were performed with native STI at 25 °C on the assumption that a simple two-state transition between native and unfolded form of the protein may occur in the disorganization process by the detergent binding. The mean residue ellipticities, $[\theta]$, were determined as a function of time, and the final equilibrium value for each experiment, $[\theta]_\infty$, was estimated by extrapolating $[\theta]$ to infinite time. When the logarithm of $([\theta]_\infty - [\theta]_0)/([\theta] - [\theta]_0)$, where $[\theta]_0$ is the value of native STI, was plotted against the time at which $[\theta]$ was measured, apparent linear plots were obtained (not shown), as required by first-order kinetics, at two wavelengths (220 and 226 nm). The apparent rate constants determined from the slopes of the plots mentioned above were 1.5×10^{-5} s^{-1} and 2.3×10^{-5} s^{-1} at $I = 0.12$ and 0.25, respectively, at the initial $NaDodSO_4$ concentration of 0.63 mM. At this concentration, the final transitioned form corresponds to the complex around $\bar{\nu} = 7$–8. The ionic strength of the medium appears to affect the rate constant as well as the position of equilibrium in STI–$NaDodSO_4$ complexing.

Effect of the Length of Hydrocarbon Chain of Alkyl Sulfates on the Transition at the First Stage. When $NaDodSO_4$ binds to intact STI around $\bar{\nu} = 5$, the positive peak at 226 nm leveled off, and a transient negative band appeared at 239 nm in the near-UV CD spectrum (curves A-2 and B-3 in Figure 2). The behavior of the former band has been regarded to reflect sharply the change of the main chain conformation (Baba et al., 1969; Koide et al., 1974). The 239-nm CD band appeared at the midpoint of STI disorganization (Tamura & Jirgensons, 1980). Therefore, the changes of these CD bands may be taken as the most sensitive index for the change of the rigidity in the structural integrity of native STI. The ability to induce these CD band changes in other detergents was investigated, and the result is shown in Figure 3. The logarithm of the minimum mole ratio, moles of detergent initially added per mole of STI, which could induce the drop of the 226-nm band nearly to zero and the appearance of the 239-nm band, was plotted against the number of CH_2 groups in the hydrocarbon chain of alkyl sulfate. The linear relationship in Figure 3 indicates that the effects of alkyl sulfates on the transition increase exponentially with the number of carbon atoms in the alkyl group of detergents. Less detergent was necessary to alter these CD bands

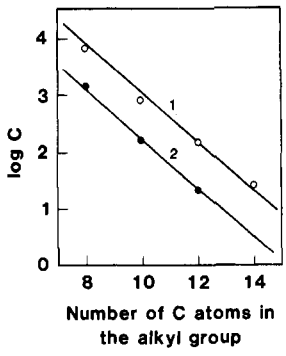

FIGURE 3: Effect of the length of hydrocarbon chain of alkyl sulfates on the transition at the first step. Log C is the logarithm of the initial minimum mole ratio, moles of alkyl sulfate per mole of STI, that causes the changes of the CD bands at 226 and 239 nm. (1) In water; (2) $I = 0.12$.

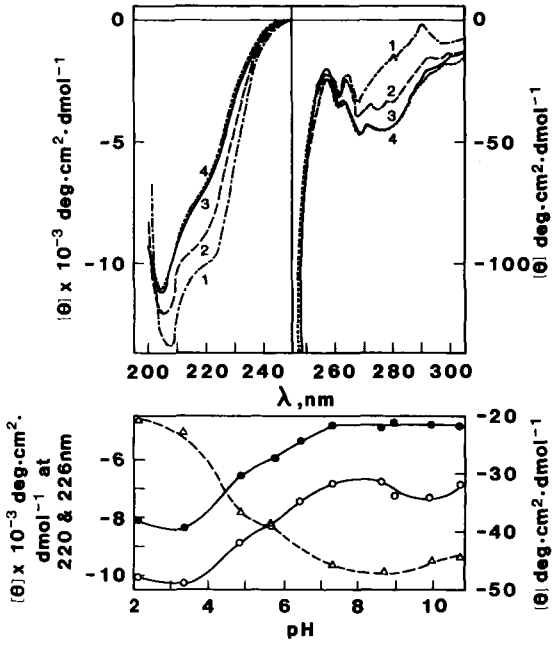

FIGURE 4: CD spectra of STI–$NaDodSO_4$ complex and mean residue ellipticities at 220, 226, and 275 nm at various pH values at $I = 0.014$. The initial mole ratio of $NaDodSO_4$ to STI is 780. (Upper) Curves 1, 2, 3, and 4 represent pH 2.1, 4.7, 7.3, and 10.8, respectively. (Lower) (○) 220 nm; (●) 226 nm; (△) 275 nm.

at $I = 0.12$ than in water. In addition, the lines in Figure 3 were almost parallel with the straight lines in a similar plot reported on the overall process of STI transitions produced by added detergent (Jirgensons & Shimazaki, 1975).

On the other hand, 8.5 M urea and 6 M guanidine hydrochloride failed to elicit any distinct CD band change, and the protein retained its native conformation in the presence of these denaturing agents in accord with the previous observations (Jirgensons et al., 1969).

pH Effects. The effects of pH on complexing of $NaDodSO_4$ with STI are shown in Figure 4. At sufficiently low pH, such as pH 3.3 or 2.1, the formation of helix was promoted appreciably as indicated by a distinct shoulder around 222 nm. Furthermore, remarkable changes appeared in the near-UV region at these pH values (Figure 4, curve 1). The negative bands centered at the 270–290-nm zone decreased drastically. The CD spectrum (Figure 4, curve 1) may represent a sort of ultimately transitioned form in the STI–$NaDodSO_4$ complex. In contrast, the near-UV CD spectra remained almost identical between pH 7.3 and 10.9. The spectra at pH 4.7 showed an intermediate form. The ellipticities at 226 and 220 nm decreased with increasing pH up to 7.3 but were not influenced at alkaline pH. Thus, the extent of transitions effected by $NaDodSO_4$ was similar in neutral and alkaline pH ranges and more complete at acidic pH.

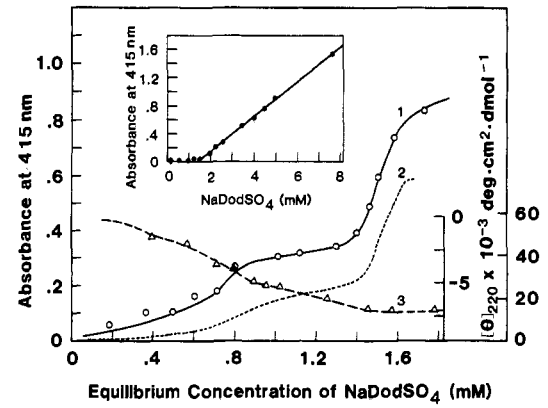

FIGURE 5: Solubilization of (dimethylamino)azobenzene by STI–$NaDodSO_4$ complex in pH 7.3 phosphate buffer ($I = 0.12$) at 25 °C. Protein concentrations are 0.1%. (1) Solubilization of (dimethylamino)azobenzene by STI–$NaDodSO_4$ complex; (2) binding isotherm at $I = 0.12$; (3) changes of $[\theta]_{220}$ in deg·cm²·dmol⁻¹. The inset shows the solubilization of the dye by $NaDodSO_4$ in the absence of the protein.

Dye Solubilization. Figure 5 represents the solubilization of a water-insoluble dye, (dimethylamino)azobenzene, by the STI–$NaDodSO_4$ complex and correlation with the binding isotherm at $I = 0.12$. As can be seen, the solubilization of the dye was parallel to the isotherm throughout the range investigated. The solubilization of the dye was little until $\bar{\nu} = 10$. The value $\bar{\nu} = 10$ is half the value reported with bovine serum albumin–$NaDodSO_4$ complex (Steinhardt et al., 1974). The solubilization was enhanced gradually as the amount of $NaDodSO_4$ binding increased. The solubilizing power abruptly increased coincidently with the rapid increase of $NaDodSO_4$ binding. It is of interest to note that helicity in STI–$NaDodSO_4$ complex, as indicated by the magnitude of $[\theta]_{220}$, reached a maximum before the steep rise of curves 1 and 2 in Figure 5. The $[\theta]$ value remained almost constant during and after the sharp increase of $NaDodSO_4$ binding and the solubilization power.

Discussion

The binding isotherms in Figure 1 consist of two distinct phases, and the conformational transitions occurring in each phase were investigated primarily by means of circular dichroism. The first phase of $NaDodSO_4$ binding (up to $\bar{\nu} = 30$ in the case of curve 2 in Figure 1) corresponds to the process of disorganization and transformation of STI into a periodic structure by the action of the amphiphile bound. The perturbation and disruption of the side-chain fine structure occurred during the initial binding stage ($\bar{\nu} = 2$–7). The conformational change of the protein backbone was also observed, as shown by the decrease of $[\theta]_{200}$ in curves A-2 and A-3 in Figure 2, but was still slight at this step. The result in Figure 3 indicates that hydrophobic interactions play a decisive role in the disorganization of STI as well as in the subsequent conformational transitions.

It is likely that the hydrophobic tails of $NaDodSO_4$ penetrate the interior of the protein, and consequently disruption of the original conformation takes place, as revealed in the studies on the effects of $NaDodSO_4$ on the X-ray patterns and volume of cross-linked lysozyme (Yonath et al., 1977).

The cooperative binding of $NaDodSO_4$ to STI began just after the initial binding step. Helical structure was gradually induced as the amount of $NaDodSO_4$ bound increased coop-

eratively in the first phase. In the second phase (more than $\bar{\nu} = 30$ in the case of curve 2 in Figure 1), sharp increase of the detergent binding occurred, and possibly maximum binding of $NaDodSO_4$ to STI was attained. No distinct conformational transition was observed during the second phase (Figures 2 and 5). Thus, in the vertical portion of the isotherms in Figure 1, the $NaDodSO_4$ association to STI may be similar to the aggregation of the detergent to micelles in the absence of the protein predominantly by the detergent–detergent interactions.

A similar dependence of binding isotherms on ionic strength of medium as shown in Figure 1 was reported with cytochrome b_5 (Robinson & Tanford, 1975) and with *S*-(carboxyamidomethyl)-κ-casein polypeptide chain (Makino & Niki, 1977) in which the cooperative binding process was regarded to resemble the cooperative formation of detergent micelles.

The binding manner of detergent molecules in the protein–$NaDodSO_4$ complex which exhibits the same dye-solubilizing ability as detergent micelles do has been presumed to be a micelle-like cluster along the protein polypeptide chain (Blei, 1960; Pitt-Rivers & Impiombato, 1968; Steinhardt et al., 1974). The importance of hydrophobic environment on the formation and stabilization of α helix has been emphasized [e.g., Tanford et al. (1960) and Timasheff & Townend (1965)]. At higher $NaDodSO_4$ concentrations, micellar $NaDodSO_4$ clusters on the polypeptide chain possibly provide a local nonpolar environment to the polypeptide chain just as in a suitable organic solvent with a small dielectric constant (Tamura & Jirgensons, 1980). However, the helix content in STI–$NaDodSO_4$ complex is not high even in completely transitioned form (A-7 in Figure 2 and curve 1 in Figure 4), if compared with that of helical proteins. It is known that the increase of helicity in such reconstructive denaturation of a protein is not invariably large upon complexing with $NaDodSO_4$ (Mattice et al., 1976; Su & Jirgensons, 1977). The reorganization of conformation depends chiefly on the primary structure (sequences of amino acids capable to form helices) and on the external conditions, such as the presence of a suitable perturbant.

Acknowledgments

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