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Alkali and Urea Induced Conformation Changes in Concanavalin A†

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ABSTRACT: The urea- and alkali-induced transitions of concanavalin A are apparently irreversible, as judged by circular dichroic (CD) spectra and hemagglutinating activity, although the nature of the product depends on the pH at which reversal is attempted. The native protein exhibits a characteristic far-uv CD spectral band centered at 223 nm, which is blue shifted by alkali and abolished by urea. Lowering pH after denaturation with alkali leads to loss of protein by precipitation in amounts dependent upon pH. The remaining soluble protein has CD characteristics which are dependent on the pH at which reversal attempts are made. A similar pH dependence of the CD spectrum occurs on dilution of urea after denaturation, but this procedure leads to little or no precipitation, perhaps as a result of the lower final protein concentrations. Reversal at pH 6 leads to a CD band centered near 223 nm; products obtained at pH 7, however, show a band at 217 nm. In this same pH interval, native Con A undergoes changes in sedimentation coefficient reflecting a transition from dimer to tetramer. Native Con A, however, exhibits a constant CD spectrum over this pH interval. Succinylated Con A has a CD spectrum closely resembling that of the native protein, but is more resistant to alkali denaturation, suggesting that specific group titration, rather than general charge effects, are responsible for the alkaline lability.

Concanavalin A, a lectin isolated from jack beans (Sumner, 1919), undergoes an apparently irreversible conformational transition between pH 8 and 9 at room temperature (Pflumm *et al.,* 1971; Zand *et al.,* 1971). A similar transition occurs at pH 8 if the protein is allowed to stand at 37° (Cunningham *et al.,* 1972). The conformational changes are followed by precipitation if the pH of the solution is below 9. Turbidity arises even in pH 7 solutions of the lectin (McKenzie *et al.,* 1972) suggesting that a slow transition occurs also at neutral pH. We have now examined this transition in greater detail in an effort to determine whether it is indeed irreversible and, if it is, what structured features are responsible. Since the conformational changes involve elements of the protein's secondary and tertiary structure, the course of the transition may be monitored by changes in the circular dichroic (CD) spectrum of Con A.

Native Con A exhibits an unusual far-uv CD band cen-

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tered at 223 nm (Kay, 1970; Pflumm *et al.*, 1971), a position ordinarily associated with α helix (Sears and Beychok, 1973). However, X-ray studies of crystalline Con A have demonstrated that more than one-third of the residues of the protein are in β-pleated sheet conformation, with only a single turn of α helix (Edelman *et al.*, 1972; Hardman and Ainsworth, 1972). Thus neglecting the remote possibility of a significant conformational difference between the crystalline and dissolved states, the distinctive CD spectrum probably arises from β structure with atypical far-uv transitions. It has been previously noted (Pflumm *et al.*, 1971) that the 223-nm band coincides in position with that of the synthetic polypeptide, poly(S-carboxymethyl-L-cysteine), which is known to exist in a β conformation (Stevens *et al.*, 1968; Ikeda and Fasman, 1967) but has CD characteristics unlike those of poly(L-lysine), the reference anti-parallel β polypeptide (Greenfield and Fasman, 1969).

During the pH-induced conformational change, the CD extremum shifts from 223 to 215–217 nm, with approximately twofold intensification (Pflumm *et al.*, 1971; Zand *et al.*, 1971). The near-ultraviolet CD bands, associated with the aromatic side chains, undergo alterations in intensity as well as in sign (Pflumm *et al.*, 1971).

The structural basis of these CD spectral changes is not understood; moreover, an investigation of functional alterations accompanying the transition, such as agglutinating activity, has not thus far been reported.

While the function, if any, of Con A in the jack bean is unknown, its well-known interaction with the erythrocyte and other animal cell membranes (Sharon and Lis, 1972; Burger and Noonan, 1970; Inbar *et al.*, 1971; Singer and Nicholson, 1972; Cuatrecasas, 1973; Edelman *et al.*, 1973; Podolsky *et al.*, 1974), when considered with its marginal stability in aqueous solution, suggests features of conformation and reactivity not shared by proteins that function primarily in aqueous media. Given the widely observed reversibility of denaturation of the latter, especially in monomeric states, and the absence of any currently accepted proposals for irreversible denaturation, it is particularly important to identify and understand irreversible conformation transitions and to determine how a particular conformational state is selected from among several accessible ones.

In this paper we report studies on the urea and alkali induced transitions of concanavalin A. To test the reversibility of denaturation, we have attempted to renature the protein at several pH values and in the presence and absence of substrate or metals. The experiments show that the pH at which refolding occurs influences the state achieved after removal of the denaturant. The pH interval over which this was expressed suggested a relationship to the known dimer–tetramer equilibrium of Con A (Kalb and Lustig, 1968), and the sedimentation behavior of native Con A was accordingly investigated to establish more precisely the pH dependence of that equilibrium. We have also sought to determine whether the transition is triggered by a change in the net charge of the protein (Akedo *et al.*, 1972), by examining succinylated derivatives. Finally, the hemagglutinating activity of the various transformed and reversed states was investigated.

Materials and Methods

Concanavalin A, isolated from defatted jack bean meal by the method of Agrawal and Goldstein (1967), was the generous gift of the laboratory of Professor G. M. Edelman of the Rockefeller University. The protein, Code No. 79-003, lot 68, was also purchased from the Miles-Yeda Laboratories division of Miles Research, Inc., Elkhart, Ind.

Alkaline Con A solutions were prepared at concentrations of 4–8 mg/ml in 0.05 M $NaHCO_3$–0.10 M NaCl (pH 9.2–9.3). The pH of these solutions was checked after the protein had been dissolved. Basic Con A solutions were back titrated by dilution with 9 volumes of phosphate-buffered saline (PBS) of the desired pH. Turbid solutions were then passed through 0.45-μ Millipore filters and the pH was adjusted if necessary.

Ultrapure urea was obtained from Mann Research Corp. and acidified prior to use (Stark *et al.*, 1960). The urea solution at pH 2 (10 M) was combined with one-tenth volume 10 X concentrated PBS and final adjustments made in the pH and volume. Concentrated PBS was prepared by dissolving 0.2 g of KH_2PO_4, 1.15 g of Na_2HPO_4, 8 g of NaCl, and 0.2 g of KCl in a final volume of 100 ml. Standard PBS contained the same reagents in 1 l. and was at pH 7.4 unless noted otherwise.

Renaturation was effected by dialysis against 100 volumes of PBS, by desalting on a Bio-Gel P-2 (Bio-Rad Laboratories, Richmond, Calif.) column (1.7 × 30 cm) in PBS (pH 7.0) or by diluting 1:50 with PBS (pH 7.0) or the same buffer containing 0.01 M methyl α-mannoside. Most frequently the experiments were performed by dilution. The CD spectrum of Con A in 0.16 M urea is essentially identical with that of the native protein and the dilution technique is the simplest.

Concanavalin A was succinylated by the method of Riordan and Vallee (1964). Approximately 50 mg of the lectin were dissolved in 5 ml of 0.15 M NaCl and placed in the cell of a Radiometer Titrator 11 connected to a Radiometer pH meter 26. After bringing the pH to about 6.9, 110 mg of succinic anhydride (Matheson Coleman and Bell) was slowly added to the protein solution while stirring. During the addition, the pH occasionally dropped to 5 despite automatic titration with NaOH. The reaction mixture was left in the pH-Stat at pH 7.0 for 30 min following the complete addition of the reagent and the end of the base uptake. It was then subjected to gel filtration on Bio-Gel P-2 to remove reagents and, except for pH adjustments, used without further treatment.

CD measurements were performed on a Cary Model 60 recording spectropolarimeter with a Model 6001 CD attachment. Experiments were generally done 2–3 times and all CD scans run in duplicate. Variations in maximum intensity were generally less than 10%. Cloudy solutions were filtered through Millipore using 0.45-μ filters prior to use. Concentrations were determined using an extinction coefficient of 1.37 (Yariv *et al.*, 1968) at 280 nm for a 1 mg/ml solution. Uv spectra were recorded on a Cary Model 14 or Model 15 spectrophotometer and, if necessary, corrected for scatter by the method of Leach and Scheraga (1960). Since renaturation is usually accompanied by precipitation, most solutions were filtered through Millipore before recording spectral data. The resulting protein concentrations were rather low ($A_{280} \simeq 0.2$) and, in some cases, the solutions still scattered light (presumably because they contained large particles in solution). Thus some concentration measurements entailed errors which could account for the observed variations in CD band intensities. These errors were minimized when renaturation was effected by dilution. The small amount of denaturant present retarded (but did not eliminate) precipitation. Measurements were made before precipitation occurred so that, in addition, concentra-

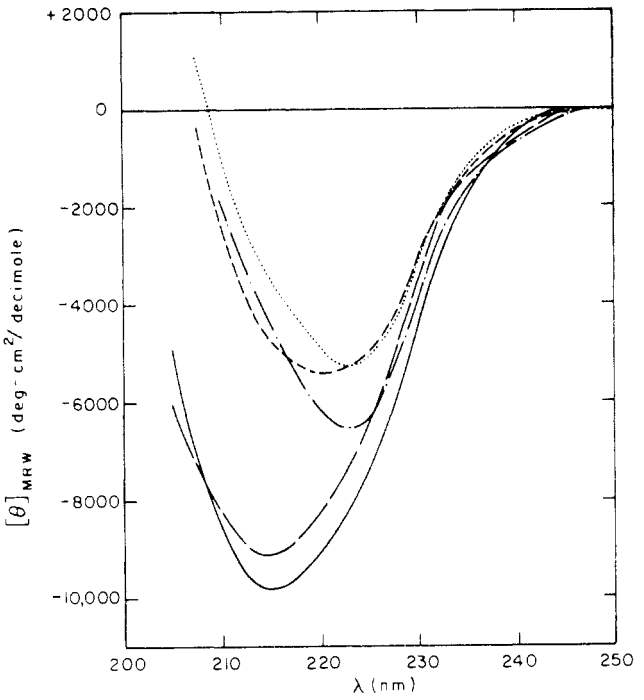

FIGURE 1: The far-uv CD band of concanavalin A: (—) in 0.05 M $NaHCO_3$–0.10 M NaCl (pH 9.3); (— —) back titrated to pH 7.2 by dilution with PBS; (- - -) back titrated to pH 6.9 by dilution with PBS; (. . .) back titrated to pH 6.1 by dilution with PBS; (• — •) native Con A, pH 6. All back titrated solutions were Millipore filtered prior to recording spectra.

tions could be determined before dilution assuming the extinction coefficient was not affected by the presence of urea.

Sedimentation velocity measurements were performed in a Spinco Model E analytical ultracentrifuge equipped with an automatic photoelectric scanner. Three samples were run simultaneously using double sector cells in a four-hole AnF rotor. The experiments were performed at 52,000 rpm and 20° with samples scanned in sequence at 8-min intervals at 280 nm (Yip *et al.*, 1972).

Hemagglutination tests were made using the defibrinated blood of mature rabbits (Pel-Freez Biologicals, Inc., Rogers, Ark.) or defibrinated sheep blood (Microbiological Associates, Bethesda, Md.) and a microhemagglutinating technique (Microtiter Instruction Manual, 1969; Kabat, 1968.)

Results

Exposure of concanavalin A to pH 9.26 results in an immediate blue shift and intensification of the protein's 223-nm CD band (Pflumm *et al.*, 1971; Zand *et al.*, 1971).

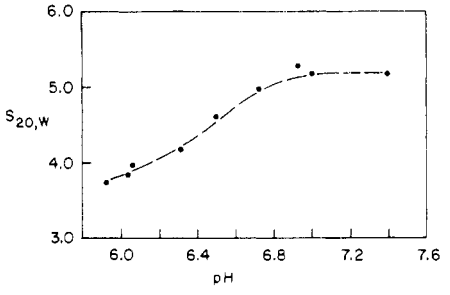

FIGURE 2: The sedimentation coefficient of native concanavalin A as a function of pH between pH 5.9 and 7.4. The buffers were in all cases PBS at the desired pH. Measurements were made at 20° using scanning optics.

TABLE I: A Comparison of End Points Observed in the Hemagglutination Assay of Con A and Its Alkali-Modified Derivatives.

Solution	Concn of Protein at End Point (mg/ml)
Back titrated to pH 6.1	2×10^{-4}
Back titrated to pH 6.9	1×10^{-4}
Back titrated to pH 7.2	6×10^{-4}
Native Con A	$3\text{-}6 \times 10^{-5}$

Back titration by dilution with PBS (pH 6.0–7.2) yields pH-dependent results. Lowering the pH to 6.1 brings about the instantaneous return of the band to its position at 223 nm but is accompanied by the loss of 85% of the protein through precipitation. In contrast, back titration to pH 7.2 is accomplished with relatively little precipitation about 15–20% and only minor changes in the altered spectrum. Reversal to pH 6.9 results in an intermediate CD spectrum with respect to band position and concomitant large losses of soluble protein. The results are summarized in Figure 1. Once concanavalin A is restored to pH 6, subsequently raising the pH to 7 does not alter its CD spectrum.

The sedimentation coefficient of the native protein was found to vary over the same pH range, as shown in Figure 2. At pH 6.1, $s_{20,w}$ was found to be 3.9–4.0 S. This increased to about 5.2–5.3 S at pH 7.0 and remained at approximately that value up to pH 7.4. At pH 9.0, native Con A has a sedimentation coefficient of 11.3 S.

Hemagglutination assays of native and alkali-modified Con A reveal diminished activity in the transformed protein. Although the agglutinating activity of the pH 7.2 and 6.1 solutions do not differ appreciably, within the rather large experimental error, both are less potent against red cells than the native lectin. Results are summarized in Table I.

Exposure to and removal of urea following pH 9 treatment do not yield a native CD spectrum. Instead the results most closely resemble those obtained after urea denaturation and renaturation (Figure 3 and discussion below). Activities are generally lower than those observed after simple back titration.

Disruption of the structure of Con A with urea followed by removal of the denaturing agent results in permanent alteration of the protein's 223-nm circular dichroism band. The data are summarized in Figures 3 and 4, and may be compared with the CD curve of native Con A shown in Figures 1 and 4.

As illustrated in Figure 3, renaturation at pH 6 yields a CD band which resembles that of the native protein much more closely than that obtained when the protein is refolded at pH 7. Following urea treatment, the blue shift at pH 7 is observed regardless of the time allowed for renaturation or the conditions employed (Figure 4). Nevertheless, an examination of the data in Table II suggests that, if anything, greater agglutinating activity is regained at neutrality than at the lower pH.

As shown in Figure 5, succinylated Con A possesses a CD spectrum which closely resembles that of the native protein. However, despite preliminary results indicating that the succinylated lectin bears at least 20 additional negative charges (M. N. Pflumm and S. Beychok, manuscript in preparation), the modified protein appears more resistant

TABLE II: A Comparison of End Points Observed in the Hemagglutination Assay of Con A and Its Urea-Modified Derivatives.[a]

Solution	Concn of Protein at End Point (mg/ml)
Renatured from urea at pH 7	$2\text{–}3 \times 10^{-3}$
Renatured from urea at pH 6	$0.8\text{–}1 \times 10^{-2}$
Native Con A	$3\text{–}5 \times 10^{-4}$

[a] The activity of the native protein differs from that obtained in the experiment above (Table I). At different times, agglutination titers of the same protein species varied considerably. This might be due to differences in the age of the cells or in cell concentrations since the cells were not counted. However, solutions to be compared were always assayed on the same day with the same batch of cells.

to alkali denaturation. The CD bands of the succinylated protein shift to shorter wavelengths at higher pH levels and are diminished rather than intensified by the presence of base.

Discussion

It has been previously shown that exposure of Con A to pH 9, or higher, leads to a conformational transition (Pflumm *et al.*, 1971; Zand *et al.*, 1971) which is not reversed by lowering the pH (Pflumm *et al.*, 1971). The present results demonstrate that there is a urea-induced transition which is not reversed by dilution at neutral pH. Addition of methyl α-mannoside or Ca^{2+} and Mn^{2+} has no apparent effect on the irreversibility of the phenomenon. Unexpectedly, attempts at reversal from either alkali or urea denaturation produce different CD spectra and, presumably, different structures when carried out at pH 6 or pH 7. Both renatured forms have diminished activity.

At pH values less than 6, Con A exists as a dimer, while at slightly alkaline pH it forms tetramers (McKenzie *et al.*, 1972; Kalb and Lustig, 1968). The limited solubility of the transformed material and experimental requirements for the scanning technique precluded direct ultracentrifugation measurements on regenerated Con A. Analysis of the data of Figure 2 reveals that the sedimentation coefficient of native Con A undergoes a sharper transition in the pH interval 6–7 than expected for a single noninteracting prototropic group. If transformed Con A behaved in the same way, the ionization state of a single side chain might determine both state of aggregation of the reformed molecule and the refolding of its subunits. It is possible, for example, that the polypeptide chain cannot assume a particular conformation while the dimer–dimer interactions remain intact.

The Con A dimer is formed by hydrogen bonding of the backbone atoms of residues 125–132 to complementary atoms of the same residues in the second chain. Tetramer formation is accomplished by interactions between side chains of the ellipsoidal dimers. In particular residues 73–78, 59–66, 48–55, 190–199, and 106–116 are involved (Edelman *et al.*, 1972; Hardman and Ainsworth, 1972). These include a single histidine, residue 51. Another histidine residue, 128, is contained in the site of monomer–monomer interaction (Edelman *et al.*, 1972).

It should be noted that the alkali-induced transition of native Con A occurs 2 pH units higher than the dimer–tetramer transition midpoint. Thus it appears unlikely that the conformational transformation could be triggered by tetramer formation or by the same side chain titration that would trigger tetramer formation. Con A contains no half-cystines (Waxdal *et al.*, 1971) and, with the exception of the N-terminus, no naturally occurring amino acid titrates normally between pH 8 and 9. Assuming that the pK of the

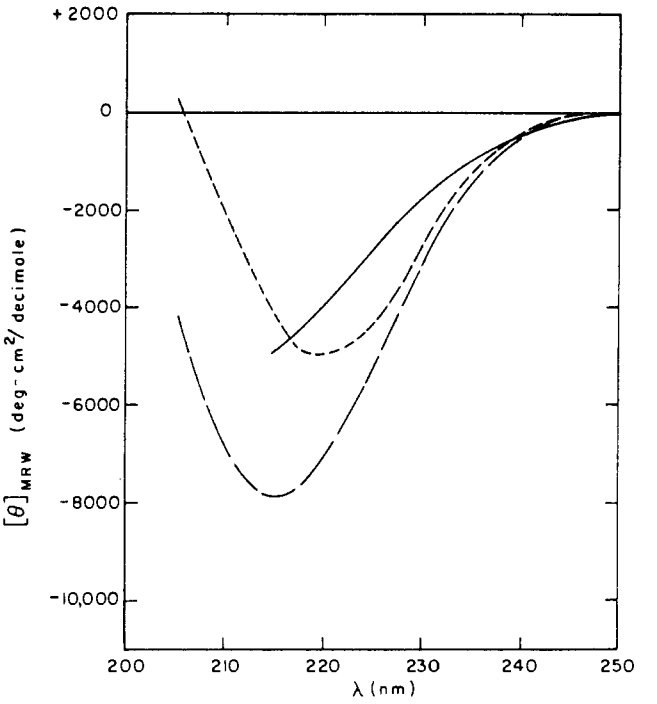

FIGURE 3: The far-uv CD band of concanavalin A: (—) in 8 M urea at pH 7.0–7.2; (— —) 8 M urea solution diluted 1:50 with PBS, pH 7.0; (---) 8 M urea solution diluted 1:50 with PBA, pH 5.9–6.0.

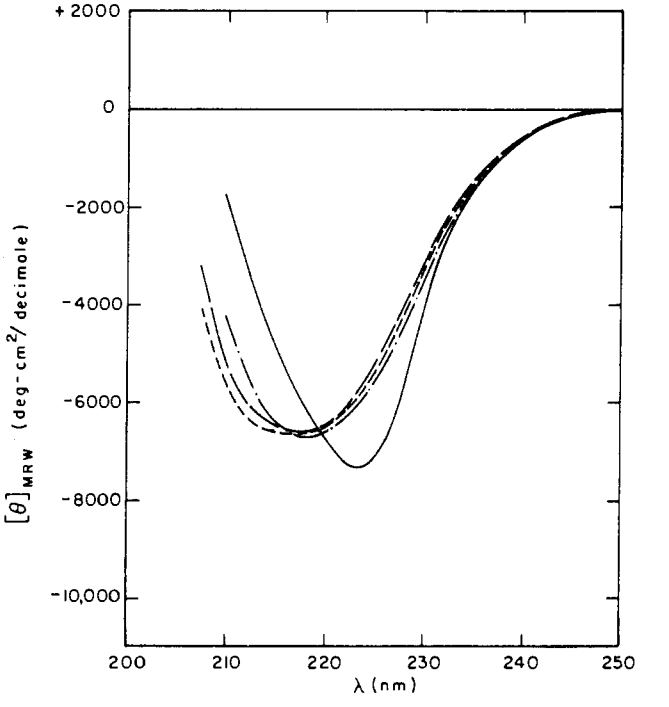

FIGURE 4: The far-uv CD band of concanavalin A: (—) freshly dissolved in 0.16 M urea–PBS (pH 7.0); (— —) 8 M urea diluted 1:50 with PBS (pH 7.0) (spectrum recorded immediately after dilution); (- - -) the same solution 19 hr after dilution; (•—•) a similar solution 19 hr after dilution with PBS (pH 7.0) containing 0.01 M methyl α-mannoside.

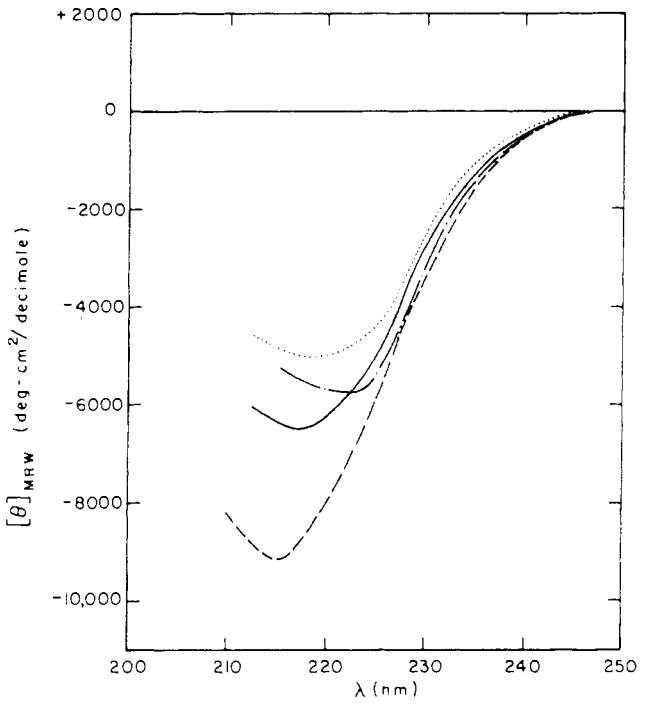

FIGURE 5: A comparison of the effects of alkali on the far-uv CD bands of native and succinylated Con A: (—) native Con A, 0.05 M Tris, and 0.10 M NaCl (pH 8.95); (- - -) native Con A, 0.05 M Tris, 0.10 M NaCl (pH 9.05); (·—·) succinyl Con A, 0.05 M Tris, and 0.10 M NaCl (pH 8.95); (. . .) succinyl Con A, 0.05 M Tris, and 0.10 M NaCl. (pH 9.05).

latter is the same as the α-NH_3^+ group of Ala-Ala-Ala-Ala (Edsall and Wyman, 1958), the N-terminus has a pK_a of about 8. It is approximately 15% uncharged at pH 7.2, 90% uncharged at pH 9, and only three residues removed from a region of β structure. A full positive charge might be required to balance the neighboring carboxylate moiety of Asp-2 or another negative charge nearby in the three-dimensional structure. Another possible cause of the transition is an abnormally reactive lysyl or tyrosyl residue. Recent studies by Hardman and Ainsworth (1972) suggest that lysyl groups with an anomalously low pK are present at the interior of the molecule. McCubbin *et al.* (1972) have suggested the presence in Con A of abnormally reactive tyrosines. A discussion of the possible effect of titrating any of these side chains on the lectin's activity must await resolution of the current uncertainty about the identification of the Con A binding site (Hardman and Ainsworth, 1973; Brewer *et al.*, 1973).

While we cannot yet identify a specific trigger for the spectral transformation, our results do suggest that net charge effects may be excluded. Although the degree of succinylation which occurs varies with the conditions employed (Gunther *et al.*, 1973) the modified protein must bear a greater negative charge. Yet rather than exhibiting increased lability, succinyl Con A shows greater alkali resistance.

The low solubility of transformed Con A at and near neutral pH complicates analysis of any equilibrium which might occur between it and native Con A. However, the earlier results of Cunningham *et al.* (1972), together with the present results, suggest that the β structure which generates the anomalous band at 223 nm is metastable in aqueous solution; mild treatments convert this structure to a more stable state which tends to form large insoluble aggregates.

Notwithstanding the apparent dependence of regeneration of the 223-nm CD band on the dimer–tetramer equilibrium, the anomalous position of the band in native Con A cannot be attributed to the β structure shared by dimers in the tetramer, since the native dimer (Kay, 1970) exhibits the same CD spectrum as the native tetramer (Pflumm *et al.*, 1971). A more likely region of the molecule is the β structure which contributes to dimer formation. The extensive sheet at that interface is atypical in geometry when compared to the skew arrangement of the backbone segments typically associated with anti-parallel segments within a single polypeptide chain (see, for example, Reeke *et al.*, 1967). It is interesting in this connection that calculated transition frequencies for anti-parallel β sheets predict the longest wavelength negative CD band to occur close to 223 nm (Madison and Schellman, 1972). While the absolute ascription of the band position to a particular geometry must be made with extreme caution, it is nonetheless likely that the anomalous CD band arises from an extensive β sheet with geometry not ordinarily observed in proteins (Sears and Beychok, 1973) and not observed in transformed Con A.

Interpretation of the agglutination results is also limited by the phase change. Since the activity of the lectin is low after transformation, it might result from the presence of untransformed Con A. Following urea treatment, the activities of protein regenerated by pH 6 and pH 7 differ unexpectedly. Although the CD spectrum of Con A refolded at pH 6 resembles that of the native protein more closely it shows less activity than Con A renatured at pH 7. Since the pH 6 CD spectrum appears intermediate between native and transformed Con A, it might represent multiple conformations. Some forms of the lectin might interfere with others in forming a matrix for precipitation, resulting in a loss of ability to agglutinate cells. Similarly, even though Con A back titrated from alkali to pH 6 shows a diminished 223-nm band, its reduced activity might be due to small amounts of inactive material preventing formation of such a matrix.

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Conformational Changes of Histone ARE (F3, III)†

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ABSTRACT: The conformational changes of histone ARE are a function of the phosphate concentration added to the solution. The conformational changes are monitored by intrinsic tyrosine fluorescence anisotropy, circular dichroism (CD), and light scattering. Histone ARE is shown to be similar to histone GRK [H. J. Li, R. R. Wickett, A. M. Craig, and I. Isenberg (1972), *Biopolymers 11,* 375] in that salt induces both fast and slow conformational changes. However, it is like histone KAS [J. A. D'Anna, Jr., and I. Isenberg (1974), *Biochemistry 13,* 2093], in that the fast step of ARE is very sensitive to changes in phosphate concentration. The fast conformational changes are characterized by increased tyrosine rigidity and α-helix formation of about 19 residues at 10^{-5} M histone. There are no slow changes below 1.5 mM phosphate. Above this critical phosphate concentration, there are time-dependent increases in light scattering, anisotropy, and CD. During this slow change, histone ARE aggregates. At the end of the aggregation there are about 11 residues of α helix and about 33 residues of β sheet per histone molecule. As with GRK, the slow changes are sensitive to temperature and histone concentration. The conformations of ARE, LAK, KAS, and GRK are summarized and compared.

This laboratory recently reported studies of conformational changes of histone GRK[1] (Li *et al.,* 1972; Wickett *et al.,* 1972; Wickett and Isenberg, 1972: Li and Isenberg, 1972; Small *et al.,* 1973; Smerdon and Isenberg, 1973, 1974), histone KAS (D'Anna and Isenberg, 1972), and histone LAK (D'Anna and Isenberg, 1974a). These studies indicated that at least part of each histone molecule has a definite folded configuration in salt solutions. The required salt concentration is, in each case, in the physiological range.

Histones GRK and KAS complex strongly with one another (D'Anna and Isenberg, 1973) as do LAK and KAS (Skandrani *et al.,* 1972; D'Anna and Isenberg, 1974b; Kelley, 1973). Upon complexing, there is a marked conformational change in one or both partner molecules (D'Anna and Isenberg, 1973, 1974b).

In the present paper, these studies are extended to an investigation of histone ARE, and a study of the complexes of ARE with other histones appears in the accompanying paper (D'Anna and Isenberg, 1974c).

A number of properties of histone ARE are known. The

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[1] The nomenclature used in this paper is described in Huberman (1973): GRK = IV = F2a1, KAS = IIb2 = F2b, LAK = IIb1 = F2a2, ARE = III = F3.