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Effect of pH, Mg^{2+}, CO_2 and Mercurials on the Circular Dichroism, Thermal Stability and Light Scattering of Ribulose 1,5-Bisphosphate Carboxylases from Alfalfa, Spinach and Tobacco

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ABSTRACT

Circular dichroism, differential scanning calorimetry and light-scattering measurements of ribulose 1,5-bisphosphate carboxylase (E.C. 4.1.1.39) from alfalfa, spinach and tobacco show: a) The conformation and thermal stability of the native carboxylases are sensitive to changes in pH and to activation of the enzyme with Mg^{2+} and CO_2. The helical content, denaturation temperature (T_d) and specific enthalpy of denaturation (Δq) decreased with increase in pH. Addition of Mg^{2+} and CO_2 at pH 9 increased T_d by 4 to 5 C; at pH 7.5 the changes in T_d were smaller. b) Addition of mercurials produced changes in conformation and thermal stability. The decrease in helical content of the enzymes with increase in pH was enhanced by the addition of *p*-chloromercuribenzoate. At pH 9, addition of *p*-chloromercuribenzoate or of 1-(3-(chloromercuri)-2-methoxypropyl)urea decreased T_d by 11.4 to 20.2 C and Δq by 2.1 to 2.8 calories per gram. c) The spinach carboxylase undergoes the largest and the tobacco the smallest changes in conformation and thermal stability upon change in pH or treatment with mercurials. d) The calorimetric data suggest that the large and small subunits are heat denatured independently but at the same temperature. e) Light scattering measurements at pH 9 of *p*-chloromercuribenzoate treated tobacco enzyme showed that there is no dissociation into subunits upon heating to temperatures greater than T_d. A 'ball and string' model for the carboxylase molecule is proposed to reconcile independence of subunit denaturation with apparent strong interactions between subunits.

Ribulose 1,5-bisphosphate carboxylases (EC 4.1.1.39) from higher plants like alfalfa, spinach and tobacco have similar molecular weights (16, 27), amino acid and subunit compositions and sequences (16, 19, 21), but show distinct differences in enzyme activity and crystallizability. The specific activity of the spinach enzyme (23, 29) is about three times that of the alfalfa enzyme (27) and six times that of the tobacco enzyme (6, 18). Carboxylases from different tobacco species show differences in their amino acid composition but have the same specific activity (31). The tobacco enzyme crystallizes readily upon dialysis against a low ionic strength Tris buffer (4). Crystallization of the spinach enzyme was only recently accomplished by Johal and Borque (15), using a vapor diffusion technique. The alfalfa enzyme has not been crystallized.

RuBPCase[1] consists of eight large (mol wt ≃ 50,000) and eight small (mol wt ≃ 12,500) noncovalently linked subunits (16). From X-ray diffraction and electron microscopic measurements, Baker, *et al.* (2) depict the molecule as two eclipsed layers of four large and four small spherical masses. Such a molecule should have minimum contact between the subunits and be readily dissociable into its subunits. However, in most cases, strong denaturants are required to separate the subunits and the isolated subunits are denatured on separation (14). Although Nishimura *et al.* (20) reported that treatment with *p*-chloromercuribenzoate at pH 9 dissociated the spinach enzyme, light scattering measurements (27) showed that the same procedure did not dissociate the alfalfa enzyme.

Results of CD, differential scanning calorimetric and light scattering measurements of the effects of pH, Mg^{2+} and CO_2, and mercurials on conformation, thermal stability and subunit cooperativity of the three carboxylases are presented in this report. In general, the measured properties of the three carboxylases are similar. There are small but significant differences in secondary structure, as revealed by CD, and in thermal stability. Mercurials markedly reduce thermal stability, but hardly affect the CD spectra and do not cause dissociation into subunits. A model is proposed to account for the observed interactions between subunits.

MATERIALS AND METHODS

The enzymes from alfalfa (specific activity = 0.45 μmol min^{-1} mg^{-1}) and spinach (specific activity = 1.5 μmol min^{-1} mg^{-1}) were prepared as previously described (7, 27). The tobacco enzyme (specific activity = 0.2 μmol min^{-1} mg^{-1}) was a gift from Professor S. G. Wildman. Enzyme concentrations were measured by applying $E^{1\%}_{1cm}$ of 15.4 at λ = 279 nm for alfalfa (27), 16.4 at λ = 280 nm for spinach (23), and 14.3 at λ = 280 nm for tobacco (6). Absorbance measurements were made at 23 C using a Cary[2] 15 recording spectrophotometer (Varian Associates, Palo Alto, CA). The pH of buffer solution was determined at room temperature. The pH of Tris buffer solution at elevated temperature is significantly lower than at room temperature.

CD spectra were obtained at 27 C with a Cary 60 spectropolarimeter equipped with a Cary 6001 CD attachment. Molecular ellipticities [Θ] in deg cm^2 $decimol^{-1}$ were calculated using mean residue weights of 110.5, 114.2 and 110.3, evaluated from the amino acid compositions of alfalfa (21), spinach (16), and tobacco (16) enzymes, respectively. Helical content was calculated using

[1] Abbreviations: RuBPCase, ribulose 1,5-bisphosphate carboxylase; CD, circular dichroism; Δq, specific enthalpy of denaturation; ΔH_d, molar calorimetric enthalpy of denaturation; ΔH_{vH}, van't Hoff enthalpy of denaturation; T_d, denaturation temperature; K_{app}, apparent equilibrium constant of the transition; n, cooperativity number.

[2] Reference to a company and/or product named by the Department is only for purposes of information and does not imply approval or recommendation of the product to the exclusion of others which may also be suitable.

equation 2c of Chen, *et al.* (5).

Differential scanning calorimetric thermograms were recorded on a DuPont model 990 thermal analyzer, calibrated and used as described previously (10). The specific enthalpy of denaturation, Δq, was determined from the area under the endotherm. A mol wt of 497,000 (27) was used to calculate the molar enthalpy of denaturation ΔH_d (= $M\Delta q$), since light scattering studies as shown herein indicated that no dissociation into subunits occurred, even on heating with mercurials. ΔH_{vH}, the van't Hoff enthalpy, was calculated from T_d (endotherm peak temperature) and $\Delta T_{1/2}$ (width of the endotherm at one-half its height) by Sturtevant's (25) formula: $\Delta H_{vH} \simeq 7\ T_m^2/\Delta T_{1/2}$, with T_d used for T_m, the temperature at which half the protein is denatured.

For a few endotherms selected because baselines could be easily estimated by eye, the endotherm shape was analyzed as a van't Hoff plot. The distance of the endotherm from the baseline was read at ½ degree intervals over the full endotherm. The 35 to 40 data points so obtained were summed to give the integral sigmoid curve for the denaturation transition. The apparent ratio of denatured to native material, K_{app}, at each temperature, was calculated from these data and a van't Hoff plot was constructed according to equations 1–3 of Freire and Biltonen (13).

The ratio of the molar enthalpy of denaturation, ΔH_d, to the van't Hoff enthalpy, ΔH_{vH}, gives n, the average number of cooperative units in the molecule of mol wt M (25). RuBPCase consists of 8 large and 8 small subunits (16) which are noncovalently linked, so that $n = 1$ indicates maximum subunit cooperativity (all subunits denature together) and $n = 16$, minimum subunit cooperativity (subunits denature independently). Pairwise (1 large and 1 small) denaturation of the subunits would give $n = 8$. Lack of cooperativity within a subunit (*e.g.*, domains which denature independently) would give $n > 16$.

Light scattering measurements were made with a Sofica (Société Francaise d'Instrumentes de Contrôle et d'Analyses, Le Mesnil Saint-Denis, S. et O., France) photometer (28). When scattering intensities were determined as a function of temperature, the experiment was started with both sample solution and the instrument thermostat at 20 C. The thermostat was then heated and scattering measurements were made as a function of time. The time—temperature relationship for the sample solution was determined in a separate dummy experiment using the same scattering cell containing the same total sample volume of buffer.

RESULTS

Circular Dichroism Measurements. The aromatic (250 to 310 nm) and intrinsic or polypeptide backbone region (200 to 250 nm) CD spectra of the three carboxylases are compared in Figure 1. The alfalfa and spinach enzymes were run in 0.01 M Tris buffer, pH 7.8. The tobacco enzyme, which tends to crystallize in low ionic strength Tris buffers (4) was run in 0.05 M K-phosphate buffer, pH 7.8. In the aromatic region the three spectra are quite similar but in the intrinsic region there are distinct differences. Increasing the pH to 9 or 10 decreased the measured ellipticities in the intrinsic region, but only small changes were observed in the aromatic region.

The effect of pH on the conformation of the native enzyme is summarized in Table I. At pH 7.8, the spinach enzyme has the highest helical content. It also shows the largest decrease in helical content with increase in pH. The tobacco enzyme has the lowest helical content and undergoes the smallest change with pH.

Scanning Calorimetry. Differential scanning calorimetric thermograms of the three carboxylases in 0.02 M K-phosphate, pH 7.5 and in 0.1 M Tris, pH 9.1, are compared in Figure 2. The actual experimental data extended from about 20 to 145 C for the alfalfa enzyme, to 135 C for the spinach enzyme, and to 120 C for the tabacco enzyme. In all cases only one endotherm was observed and both T_d and Δq (except for the tobacco enzyme, Table II) were lower at the higher pH.

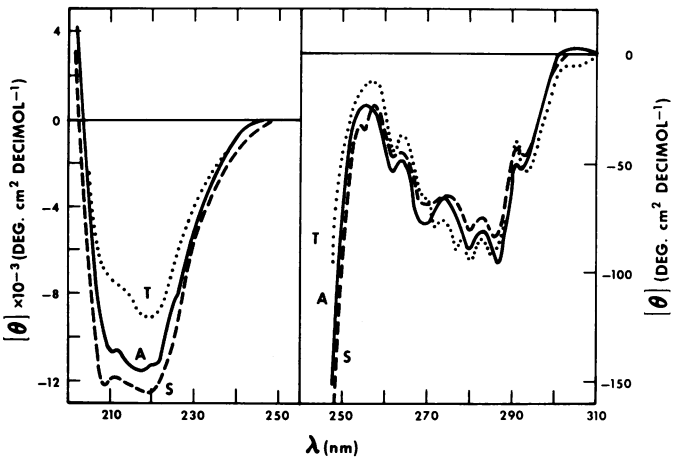

FIG. 1. Aromatic (250–310 nm) and intrinsic (200–250 nm) region CD spectra for alfalfa (——) and spinach (- - -) enzymes in 0.01 M Tris-HCl, pH 7.8 and for tobacco (· · ·) enzyme in 0.05 M phosphate, pH 7.8 buffers. Experimental conditions: aromatic region, 0.04 degree scale, 1.0 cm path length and concentrations of 1.089, 1.111 and 1.133 mg/ml for alfalfa, spinach and tobacco enzymes, respectively; intrinsic region, 0.04 degree scale, 0.05 cm path length and concentrations of 0.371, 0.380 and 0.378 mg/ml for alfalfa, spinach and tobacco enzymes, respectively.

Table I. *Effect of pH on the Conformation of Native and p-Chloromercuribenzoate-treated Ribulose 1,5-bisphosphate Carboxylase from Alfalfa, Spinach and Tobacco*

Alfalfa and spinach enzymes CD spectra were run in 0.01 M Tris, the tobacco enzyme in 0.05 M K-phosphate. See legends to Figures 1 and 3 for representative experimental conditions. The mercurial-treated enzyme solutions were prepared by adding sufficient 0.04 M *p*-chloromercuribenzoate to inactivate all of the 90 sulfhydryl groups per mole (16) and provide a 10% excess.

Enzyme	pH	Per cent Helix		
		Alfalfa	Spinach	Tobacco
Native	7.8	29	32	21
	9.0	26	27	21
	10.0	24	24	18
p-Mercuribenzoate-treated	7.8	29	28	20
	9.0	24	18	16
	10.0	16	15	16

The effects of buffer and pH are summarized in Table II. The thermal stability data for all three enzymes appear to show a large pH effect, T_d being smaller by 3 to 8 C at the higher pH. T_d (not listed in Table II) for the spinach enzyme in the same buffer system (0.05 M Tris) but at different pH (7.8 and 9.1) showed a much smaller change (0.5 C). This suggests that the temperature stabilization at pH 7.5 in phosphate buffer could be largely due to a specific effect of phosphate and only partly due to the lower pH. A similar specific stabilizing effect of phosphate has been reported for aspartate transcarbamoylase (30).

The three transitions selected for a detailed analysis of the shape of the endotherm (alfalfa in phosphate, pH 7.5, spinach and tobacco in Tris, pH 9) gave excellent linear van't Hoff plots between the limits 1.5% denatured and 98.5% denatured protein (8 to 9 units of ln K_{app}). Points outside this range deviated from the straight line; these deviations appeared to result from small errors in estimation of the baseline.

Effect of Mercurials. Treatment of the alfalfa enzyme with *p*-chloromercuribenzoate at pH 7.8 produces only small changes in the intrinsic region CD, but large changes occur in the aromatic

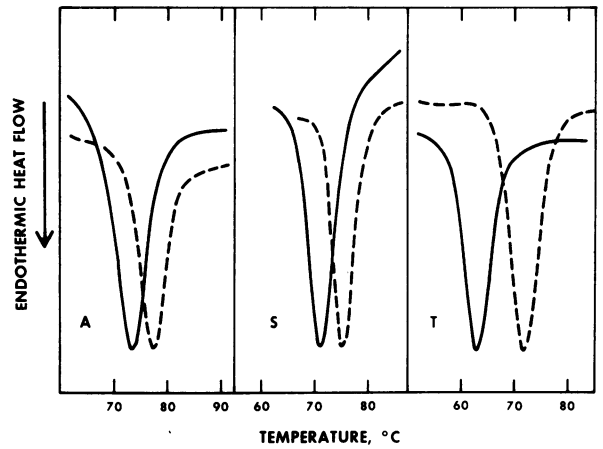

FIG. 2. Differential scanning calorimetric thermograms of alfalfa (A), spinach (S) and tobacco (T) enzymes in 0.02 M phosphate, pH 7.5 (- - -) and in 0.1 M Tris-HCl, pH 9.1 (——) obtained at a heating rate of 10 C/min. Enzyme concentrations at pH 7.5 are 2.25×10^{-5}, 1.38×10^{-5} and 5.01×10^{-5} M, and at pH 9 are 3.98×10^{-5}, 3.88×10^{-5} and 5.71×10^{-5} M for alfalfa, spinach and tobacco, respectively. Thermograms have been roughly scaled to the same size.

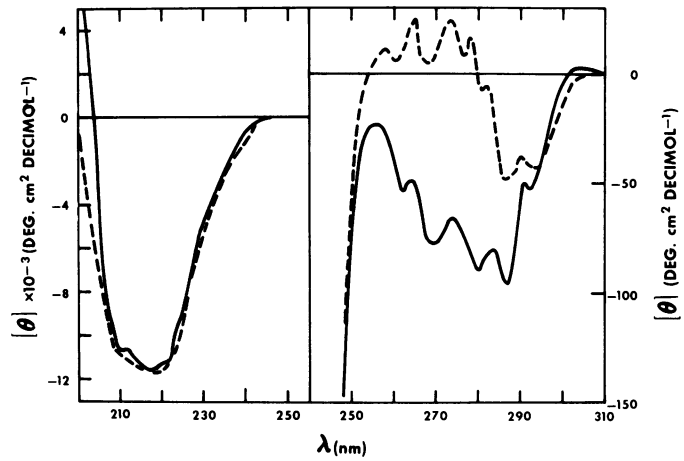

FIG. 3. Aromatic (250–310 nm) and intrinsic (200–250 nm) region CD spectra of native (——) and *p*-chloromercuribenzoate treated (- - -) alfalfa enzyme in 0.01 M Tris-HCl, pH 7.8. Experimental conditions: aromatic region, 0.04 degree scale, 1.0 cm path length, 1.089 mg native enzyme/ml and 1.083 mg mercurial-treated enzyme/ml; intrinsic region, 0.04 degree scale, 0.05 cm path length, 0.371 mg native enzyme/ml and 0.369 mg mercurial-treated enzyme/ml.

region (Fig. 3). Similar CD results were obtained for the mercurial treated spinach and tobacco enzymes.

Thermograms for native, *p*-chloromercuribenzoate treated and 1-(3-(chloromercuri)-2-methoxypropyl)-urea treated enzymes in 0.1 M Tris pH 9.1 are shown in Figure 4. When an excess of mercurial was added, a large decrease in both T_d and ΔH_d was observed for all three enzymes. Binding of *p*-chloromercuribenzoate decreased T_d by 11.4 to 17.9 C (Table II). The spinach enzyme shows the largest decrease in T_d and the tobacco enzyme the smallest. The mercury derivative of urea caused larger (14.3 to 20.2 C) decreases in T_d. When limited amounts of mercurials were added, two endotherms were observed, one for native protein and one characteristic of the fully reacted protein. No intermediate endotherms were observed.

Calculated values of the cooperativity number, *n* (ratio of the calorimetric enthalpy to the van't Hoff enthalpy) in Tris at pH 9, and phosphate at pH 7.5, with and without Mg^{2+}, CO_2, or mercurials are given in Table II.

Light Scattering Measurements. Light scattering measurements (27) showed that at 23 C, the mercurial treated alfalfa enzyme does not dissociate at pH 9; similar light scattering results (not shown) were obtained for the *p*-chloromercuribenzoate treated spinach enzyme at room temperature. Possible dissociation into subunits during heating of the mercurial treated tobacco enzyme at pH 9 was checked by light scattering (Fig. 5). Similar results were obtained at an enzyme concentration of 0.32 mg/ml at a slower heating rate (450 min to reach 49.3 C). These results indicate that dissociation of the enzyme into subunits did not occur even after heating above T_d (51.6 C). The approximately two-fold increase in scattering between 38.5 and 54.5 C indicates that aggregation occurred, but that aggregation, on the average, did not go beyond formation of dimers. The increase in scattering occurred without any discontinuity in the i_{90} *versus* temperature plot (Fig. 5). Complete dissociation into subunits should have resulted in a 92% decrease in scattering. This decrease in scattering due to dissociation could not be entirely masked by aggregation, and it could not have occurred without producing some irregularity in scattering as a function of temperature. Accordingly, the enzyme cannot have dissociated into subunits over the temperature range of denaturation.

DISCUSSION

Native Enzyme Conformation. The three carboxylases have helical contents of 20–30%. Although the helical contents of proteins calculated from CD measurements may not be exact (5), they are generally in reasonable accord with helical contents determined from X-ray structures. Differences in helical content between the carboxylases should be significant, and relative changes in CD produced by changes in pH should also be significant. The decreases in calculated helical content with increasing pH (Table I) are plausible, since the isoelectric pH is near 4.5 (1). The increase in net charge should destabilize ordered conformations. A change in enzyme conformation with increasing net charge might be related to enzyme flexibility and specific activity. Less flexibility might favor crystallization of the enzyme. The spinach enzyme shows the largest change in CD with pH; the tobacco enzyme, the smallest.

Thermal Stability of the Native Enzymes. The single thermal transition observed in these differential scanning calorimetry experiments appears to be the denaturation of the entire RuBPCase 16-unit assembly. The enthalpy of the transition is typical of protein denaturation. Thermal precipitation of spinach and tobacco RuBPCases has been observed to occur at 70 C in isothermal experiments (1). When maize RuBPCase was partly heat-precipitated in Tris-HCl at pH 7.4, both large and small subunits were found in both the precipitate and in the supernatant solution (1).

The linearity of the van't Hoff plots for the transitions of the three enzymes strongly suggests a two-state (all or none) denaturation; both subunits appear to be denatured simultaneously. However, reversibility of the denaturation transition could not be demonstrated on cooling and reheating. At the relatively high concentrations of protein required for these experiments (11–45 mg/ml), aggregation of unfolded protein at higher temperature probably prevents renaturation on cooling.

The decreases in thermal stability of the carboxylases, as reflected in T_d, appear consistent with the decreases in helical content with increase in pH (Table I). There seems to be a larger effect than might be expected from the relatively small change in helical content observed between pH 7.8 and 9.0.

Addition of Mg^{2+} and CO_2, which are required to activate the enzyme, produces a large temperature stabilization in Tris buffer (Table II). Most of the stabilization appears to be due to Mg^{2+}. The heat stability of *Cyanidium caldarium* RuBPCase was also enhanced by Mg^{2+}, much more so than by CO_2 (12). In phosphate, smaller changes in T_d occur; for tobacco there is a small decrease.

Table II. Effect of buffer, pH, Mg^{++}, CO_2 and mercurials on T_d, Δq, ΔH_{vH} and n for ribulose 1,5-bisphosphate carboxylases from alfalfa, spinach and tobacco.

Experimental conditions: see legend to Fig. 2 for representative experimental conditions used to obtain data on the native enzyme and legend to Fig. 4 for data on the mercurial-treated enzymes; in determining the effect of Mg^{++} and CO_2, a typical sample solution (alfalfa, pH 7.5 data) was prepared by mixing 15 µl of 4.52×10^{-5}M alfalfa enzyme, 5 µl of 0.02M $MgSO_4$ and 2 µl of 1M Tris - CO_2.

	In 0.02M Phosphate, pH 7.5		In 0.1M Tris, pH 9			
Enzyme Source	Native	Mg^{++} and CO_2 Added	Native	Mg^{++} and CO_2 Added	p-chloromercuri-benzoate added	1-(3-(chloromercuri)-2-methoxypropyl)-urea Added
			Denaturation Temperature, °C			
Alfalfa	76.2	77.9	72.9	76.8	57.9	52.7
Spinach	74.1	75.1	68.9	74.1	51.0	50.0
Tobacco	70.6	70.0	63.0	68.0 (67.0[a])	51.6	48.7
			Specific Enthalpy of Denaturation, cal/g[b]			
Alfalfa	6.3	6.5	5.0	5.5	2.4	2.2
Spinach	5.1	5.4	4.3	4.2	1.6	1.7
Tobacco	4.1	3.9	4.1	3.8	1.6	2.0
			van't Hoff Enthalpy of Denaturation, kcal/mol[b]			
Alfalfa	158	131	122	123	78	66
Spinach	192	179	136	167	49	64
Tobacco	137	129	132	133	107	100
			Cooperativity Number, $n = M \Delta q / \Delta H_{vH}$[c]			
Alfalfa	20	25	21	22	15	17
Spinach	13	15	15	12	16	14
Tobacco	15	15	16	14	8	10

[a] Mg^{++} only.

[b] Std. Dev. (σ_{n-1}) averaged 7% of the measurement.

[c] Derived Std. Dev. is ± 10% of n. Thus, differences of approximately 2 to 3 in n appear to be within experimental error.

The enzyme is apparently stabilized by phosphate. Stabilization and activation must be two distinct processes. Both phosphate and Mg^{2+} can increase heat stability but only Mg^{2+} can activate the enzyme.

Effect of Mercurials. The mercurial, *p*-chloromercuribenzoate is often used to react with the free sulfhydryls of the carboxylase (20). It is of interest to know whether the mercurial produces changes in conformation or stability, because it has been suggested that the binding of *p*-chloromercuribenzoate leads to separation of the spinach enzyme subunits (20). At pH 7.8 (Table I), there is little effect on the conformation of the alfalfa and tobacco enzymes. The spinach enzyme shows a distinct decrease in helical content. Increasing the pH causes a greater decrease in helical content of the mercurial treated enzymes than of the native enzymes. Thus, *p*-chloromercuribenzoate binding decreases the stability of the helical conformation of all three enzymes toward an increase in pH. Like the native enzyme, the mercurial treated spinach enzyme is least stable. In the presence of *p*-chloromercuribenzoate, all three carboxylases have nearly equal helical content at pH 10.

The aromatic region CD is largely determined by the conformational environment of the side chain chromophores (24). Thus, the large change observed upon treatment with mercurial (Fig. 3) deserves comment. There appears to be little change in the fine

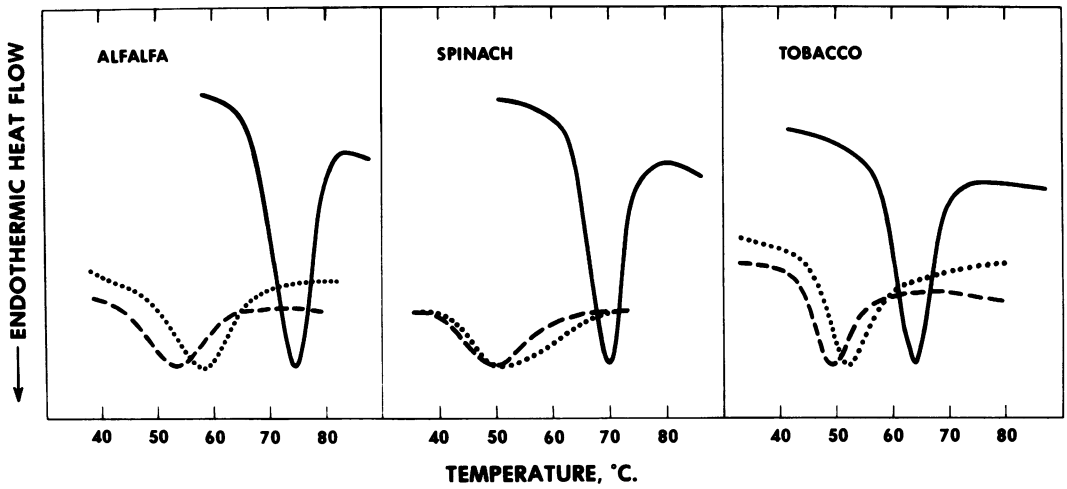

FIG. 4. Differential scanning calorimetric thermograms of native (——), *p*-chloromercuribenzoate-treated (···) and 1-(3-(chloromercuri)-2-methoxypropyl)-urea-treated (- - -) alfalfa, spinach and tobacco enzymes. Experimental conditions: 10 C/min heating rate, 0.1 M Tris-HCl, pH 9; concentrations of enzymes ranged from 1.5- 8 × 10^{-5} M, of mercurials, 2- 10 × 10^{-3} M.

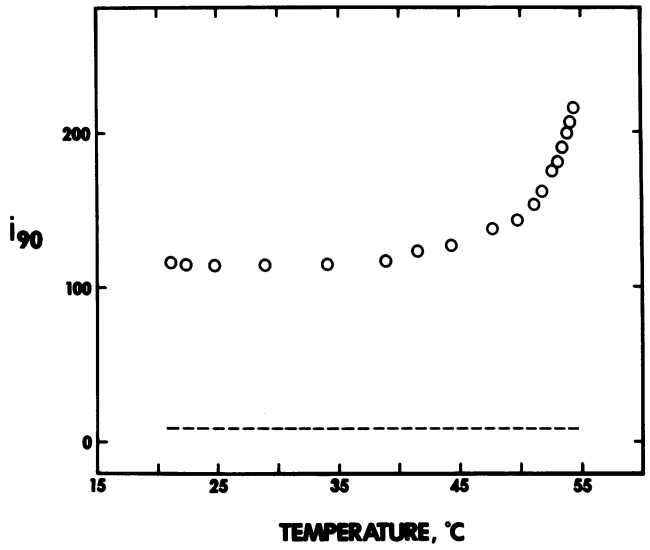

FIG. 5. Effect of heating *p*-chloromercuribenzoate treated tobacco enzyme at pH 9 on the intensity of light scattered at an angle of 90 degrees to the incident beam, i_{90}. Sample solution (10.0 ml) contained 0.277 mg enzyme/ml buffer (0.1 M Tris-HCl, pH 9) plus 0.015 ml of 0.05 M *p*-chloromercuribenzoate. Total time for the experiment was 76 min. ---, the expected scattered light intensity for complete dissociation of the enzyme into subunits in the absence of aggregation.

structure; the main change is in the magnitude of the broad negative band between 250 and 290 nm. Since the enzyme does not have disulfide bonds (17), the disulfide chromophore is not involved. The sulfhydryl group does not have an absorption band in this region of the spectrum, however, when *p*-chloromeribenzoate reacts with a sulfhydryl group, a weak absorption band at 250 nm is produced (3). If the chromophore responsible for this absorption band produces positive ellipticities of comparable magnitude over most of the same wavelength range as the broad, negative band of the native enzyme, then a cancellation could occur and produce the observed spectrum. This interpretation of the aromatic CD changes in Figure 3 suggests that no change in conformational environment of the aromatic chromophores is produced by reaction of the enzyme with *p*-chloromercuribenzoate but that some of the resulting -*S*-mercuribenzoate groups introduced into the enzyme are located in asymmetric environments.

The greater than 50% decrease in enthalpy of denaturation, Δq, for all three enzymes suggests that binding of mercurial produces large changes in enzyme structure. The CD data (compare helical content at pH 9, with and without the mercurial, Table I) show comparatively small changes in helical content particularly for the alfalfa enzyme. Similar large decreases in T_d and Δq occur upon removal of metal from diferric-conalbumin at pH 7.5 (11); CD measurements (26) indicate that the secondary structure of the apoconalbumin is unaffected by iron binding. Thus, binding of mercurials by the carboxylases and removal of iron from diferric conalbumin must produce changes, *e.g.*, a general loosening of the molecular structure, which results in large decreases in T_d and Δq but only small or no change in the secondary structure as measured by CD.

Subunit Cooperativity. Although RuBPCase consists of two distinct subunit types (16), only one thermal transition was observed. It does not seem likely that one type of subunit remained native upon heating (1) or that one subunit type had a very low enthalpy of denaturation or a very broad denaturation range, and so was not observed to denature in the calorimeter. It seems most likely that the two types of subunit were denatured together as a unit; the subunits could not be heated separately since the SDS treatment usually used to separate the subunits (14) causes denaturation. This suggests a high degree of cooperativity between the two types of subunit.

Various degrees of cooperativity in proteins have been observed by differential scanning calorimetry. Association of proteases and inhibitors give complexes which show only one endotherm (9) although the separated proteins have different T_d. Within a single chain protein, domains may give rise to separate endotherms (8, 22). Regulatory and catalytic subunits produce separate endotherms (30). The present case appears different from these three examples. The cooperativity numbers in 0.02 M K-phosphate, pH 7.5, (Table II) suggest that, for all three enzymes, there is minimum cooperativity, and the subunits denature independently. Since only one endotherm is observed, the two types of subunits must have identical or nearly identical T_d. An *n* larger than the number of subunits for the alfalfa enzyme could be due to subunit domain structure. Calculation of ΔH_{vH}, hence *n*, assumes that denaturation is a two state process, A ⇌ B (25). If the transition is more complex, *e.g.*, A ⇌ B → C, or if the subunits differ slightly in T_d, the transition will be broadened and *n* evaluated assuming a two-state process will be too large. As stated above, van't Hoff plots for endotherms of each of the three carboxylases were linear, suggesting that the transition is two state.

Neither increasing the pH to 9 in Tris buffer nor adding Mg^{2+}

and CO_2 has an effect on n (Table II). Thus, changes in conformation and changes in thermal stability due to the indicated change in pH and the binding of Mg^{2+} and CO_2 have no effect on subunit cooperativity. Reaction of free sulfhydryls with mercurials appeared to increase cooperativity for the alfalfa and tobacco enzymes. Conceivably, the partial unfolding of polypeptide chain indicated by the decrease in helical content at pH 9 (Table I) could lead to greater interaction between subunits. Cooperativity of the spinach enzyme appears to be unaffected by mercurials. The endotherms for the mercurial treated spinach enzyme (Fig. 4) were asymmetric suggesting that transition is more complex than a two state process.

The observation of a single endotherm for denaturation of RuBPCase under a variety of conditions, coupled with failure of light scattering measurements to show dissociation, suggests that there are strong interactions between subunits. However, the calorimetric experiments also suggest minimum cooperativity between subunits of the native enzymes. These conclusions are difficult to reconcile. They seem to require a 'ball and string' model for RuBPCase. In this model, compact subunits are assembled together almost without interaction or contact between subunits, in agreement with the X-ray model (2), but at the same time with the subunits strongly attached to one another by short, nondenaturable links. Such a structure might arise from the folding or entanglement of a portion of one subunit into the structural region of another, so that separation requires considerable alteration of subunit conformation. It is evident that further efforts to separate the subunits by nondenaturing methods, or to renature the subunits after separation, should be carried out.

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