Interaction of Cibacron Blue F_3GA with Glutamine Synthetase: Use of the Dye as a Conformational Probe. 1. Studies Using Unfractionated Dye Samples†

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ABSTRACT: Cibacron Blue F_3GA dye has been used to probe subtle conformational changes in protein structure associated with the conversion of *Escherichia coli* glutamine synthetase (GS) between relaxed, taut, oxidized, and dissociated forms. Binding of the dye to each form of the enzyme elicits a different spectral perturbation of the dye which can be detected by difference spectroscopy. By following time-dependent changes in the difference spectrum associated with the binding of dye to the enzyme, it was demonstrated that dissociation of subunits provoked either by urea or by relaxation of the enzyme at pH 8.5 is a multiphasic process. In the presence of 3–4 M urea, dissociation of taut GS is associated with an almost instantaneous, transient increase in absorbancy of the difference spectrum at 638 nm and, after a lag, by a progressive decrease in absorbancy at 585 nm and an increase at 700 nm. The kinetics of these changes vary as a function of temperature, pH, and the concentrations of KCl, $MnCl_2$, and urea, probably reflecting differences in the rates of GS relaxation and in the formation of aggregates of intermediate sizes. Results of direct binding measurements show that the taut and relaxed forms of GS can bind only 1–1.3 equiv of dye per subunit, whereas dissociated subunits bind up to 3.0 equiv per subunit. The K_d of the dye–taut GS complex as calculated from binding data was 0.55 μM. The binding of dye to taut GS was inhibited by its substrate, ADP, and by the allosteric effectors AMP and tryptophan. On the basis of the abilities of ADP, AMP, and tryptophan to inhibit the binding of dye to GS, dissociation constants of the respective GS–ligand complexes were 2.4, 121, and 1170 μM, respectively, in good agreement with previously determined values. From the difference spectra obtained between a given concentration of dye in a 5.0-cm cell and 10 times that concentration in a 0.5-cm cell, it was established that at concentrations greater than 5 μM a significant fraction of the dye is present as stacked aggregates. Because only the dye monomer binds to GS, the difference spectrum between dye and dye bound to GS is due in part to GS-promoted shifts in the equilibrium between stacked and unstacked dye molecules. Consequently, with increasing dye concentrations, the amplitude of the dye vs. dye + GS difference spectrum can continue to increase, even after the GS becomes saturated with dye. This stacking phenomenon precludes reliable estimation of the K_d of the GS–dye complex from spectral titration data. Urea, dioxane, and Lubrol WX suppress dye stacking, whereas high salt concentrations favor stacking. It was concluded that differences in the spectral perturbation between various forms of GS are due to variations in the stoichiometry of dye binding and in the microenvironment of the binding sites and to shifts in the dye stacking equilibrium.

Cibacron Blue F_3GA has been used extensively in the preparation of insoluble matrices for affinity chromatography of enzymes possessing nucleotide binding sites [for reviews, see Stellwagen (1977), Thompson et al. (1975), and Burgett & Greeley (1977)]. Because various kinases, dehydrogenases, and synthetases are selectively adsorbed to insoluble Cibacron Blue matrices and can be selectively eluted therefrom by various nucleotides (Burgett & Greenley, 1977; Ryan & Vestling, 1974; Chambers & Dunlap, 1979; Böhme et al., 1972; Stellwagen, 1976; Edwards & Woody, 1979), it was proposed (Thompson et al., 1975; Thompson & Stellwagen, 1976; Stellwagen, 1977) that the dye serves as a nucleotide analogue and binds to the "dinucleotide folds" of these enzymes.

Thompson & Stellwagen (1976) showed further that the binding of Cibacron Blue F_3GA to lactate dehydrogenase and phosphoglycerate kinase is accompanied by a red shift in the absorption spectrum and that this could be made the basis of a spectrophotometric method for quantitative measurements of the nucleoside phosphate binding sites. Because a similar red shift in the spectrum is obtained when the dye is dissolved in organic solvents, they suggested that the dye binds to hydrophobic pockets designed to bind the aromatic rings of NAD or NADH. This interpretation was supported by the fact that these nucleotides could quench the dye vs. dye + enzyme difference spectrum, presumably by displacing the dye from the enzyme. Although the nucleotide fold hypothesis is likely correct for many enzymes, the generality of the concept has been questioned. Cibacron Blue has been shown to inhibit some kinases and dehydrogenases that do not possess a dinucleotide foldlike structure (Beissner & Rudolph, 1978; Wilson, 1976), and a number of structurally dissimilar dyes have been shown to inhibit lactate dehydrogenase (Ashton & Polya, 1978), and a protein which does have a dinucleotide fold does not bind Blue Dextran Sepharose (Beissner et al., 1979). The possibility that dye binding can occur nonspecifically to hydrophobic regions on proteins is supported by other studies (Glazer, 1970; Anderson & Reynolds, 1965; Beissner & Rudolph, 1978; Seeling & Colman, 1977). Meanwhile, Subramanian & Kaufman (1980) presented evidence that differences in the difference spectra associated with

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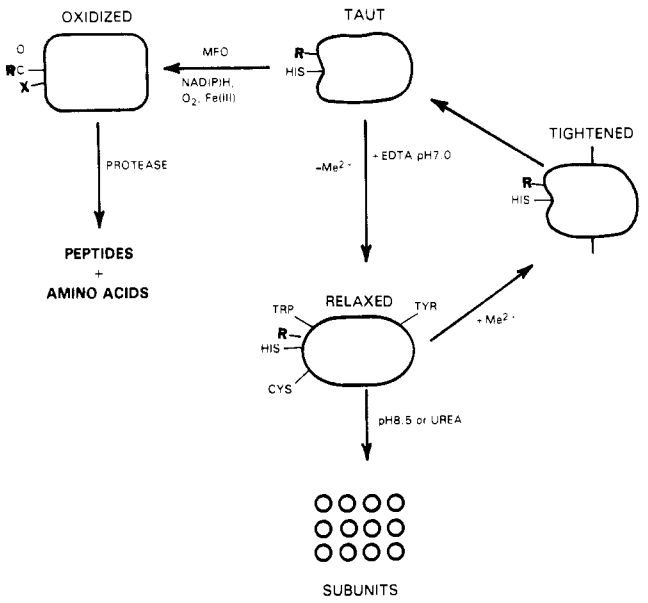

FIGURE 1: Schematic representation of the interconversion of taut, relaxed, tightened, and oxidized forms of GS.

the binding of Cibacron Blue to the dihydrofolate reductases from chicken liver and *Lactobacillus casei* could be explained by differences in the contributions of hydrophobic and ionic interactions at the nucleotide binding sites.

Whatever the mechanism of binding, the glutamine synthetase (GS)[1] from *Escherichia coli* should provide a good system to examine the possibility that Cibacron Blue F_3GA can be used as a spectral probe to differentiate between different conformational states of an enzyme. As normally isolated, this enzyme exists as an aggregate (M_r 600 000) of 12 identical subunits arranged in 2 hexagonal layers (Valentine et al., 1968; Woolfolk et al., 1966; Ginsburg, 1972) and contains 3–4 equiv of Mn^{2+} per subunit (Shapiro & Stadtman, 1967). The relationship between this native, catalytically active form of the enzyme and other well-characterized forms is illustrated in Figure 1. In the native, so-called "taut" configuration, the enzyme is very stable; all four of the sulfhydryl groups in each subunit are buried and are inaccessible to reaction with organic mercurial or other conventional sulfhydryl reagents. Upon removal of the divalent cations by treatment with EDTA, or by passage of the enzyme over Chelex ion-exchange resin, the enzyme is converted to a catalytically inactive "relaxed" configuration (Shapiro & Ginsburg, 1968; Kingdon et al., 1968; Hunt & Ginsburg, 1980). Although the molecular weight remains unchanged, "relaxation" of the enzyme involves distortion of the native quaternary structure which is disclosed by alterations in the viscosity and sedimentation coefficient, as well as by exposure of sulfhydryl groups, and by a perturbation of the ultraviolet absorption spectrum, reflecting exposure of aromatic amino acid residues (Ginsburg, 1972). The relaxed enzyme is easily dissociated by increasing the pH or by addition of low concentrations of denaturants (Shapiro & Stadtman, 1967), whereas the native taut enzyme is resistant to these treatments. With the addition of divalent cations, the relaxed enzyme is converted back to a "tightened" form that is identical with the native taut enzyme with respect to known physical, chemical, and catalytic properties, except that the tightened enzyme precipitates from dilute salt solutions as paracrystalline aggregates. This implies that the tightened enzyme is not structurally identical with the native, taut, form of GS. Figure 1 shows also that when GS is exposed to NAD(P)H, O_2, Fe(III), and any one of several mixed-function oxidation (MFO) systems, a single histidine residue in each subunit is oxidized to an unidentified derivative; in addition, 1 equiv of carbonyl group is formed (Levine, 1983). This "oxidized", catalytically inactive form of the enzyme is very susceptible to degradation by some proteases (Levine et al., 1981; Oliver et al., 1982).

In this report, we demonstrate that Cibacron Blue F_3GA can be used to monitor structural alterations in proteins by means of difference spectroscopy. We show also that at concentrations greater than 5 μM Cibacron Blue exists partly in a stacked state and that destacking of the dye is partly responsible for the spectral perturbation observed upon binding to GS. In the following paper (Federici & Stadtman, 1985), we show that Cibacron Blue F_3GA is a heterogeneous mixture of dyes which interact differently with the various known structural forms of the protein.

Materials and Methods

Cibacron Blue F_3GA was obtained from Ciba Geigy and Pierce Chemical Co. The dye concentration was measured spectrophotometrically at 610 nm with an extinction coefficient of 13.6 mM^{-1} cm^{-1} (Thompson et al., 1976). All chemicals were of reagent grade. Solutions were prepared by using distilled, deionized water and ultrafiltered through sterile Millipore filters (0.45 μm). Ultrapure reagent-grade urea was obtained from Schwarz/Mann, Inc., and purified by passage over a mixed-bed ion-exchange resin.

Glutamine Synthetase Preparations. Unadenylylated glutamine synthetase ($GS_{\overline{1-2}}$) was purified from *E. coli* W (Miller et al., 1974) and also prepared by phosphodiesterase treatment of GS6 (Rhee et al., 1976). Adenylylated glutamine synthetase ($GS_{\overline{11}}$–$GS_{\overline{12}}$) was prepared by adenylyltransferase-catalyzed adenylylation, in vitro, of GS6 according to conditions described previously (Rhee et al., 1978). All glutamine synthetase preparations gave activities of 95–115 units/mg in the Mn^{2+}, pH 7.57, γ-glutamyl transfer assay at 37 °C. The average state of adenylylation ($\bar{n}$) was determined by measurement of activity in the presence of 0.4 mM Mn^{2+} and also in the presence of 0.4 mM Mn^{2+} plus 60 mM Mg^{2+} (γ-glutamyl transfer assay in dimethylglutarate–triethanolamine at pH 7.57) (Stadtman et al., 1979). Protein concentration was determined by UV spectrophotometric measurements using published extinction coefficients and a light-scattering correction (Ginsburg et al., 1970). Stock protein solutions for difference spectra were prepared by dialyzing the enzyme at 4 °C against 10 mM imidazole (pH 7.0) (~24 °C), 100 mM KCl, and 1 mM $MnCl_2$. The final dialysate was used for the appropriate additions to the reference cuvette.

Difference Spectra. Dye vs. dye + GS difference spectra were obtained with a Cary 219 spectrophotometer (Varian Instruments) using semimicro cells (10-mm path length). Spectra were recorded at 25.0 ± 0.5 °C unless otherwise noted. Generally, 0.9 mL of the dye reaction mixture was placed in the sample and reference cuvettes, and the base line was recorded and corrected by using the auto-base-line correction function of the instrument. Identical increments of either stock protein solution or final dialysate were added to the sample and reference cuvettes, respectively, and the difference spec-

[1] Abbreviations: GS, glutamine synthetase [L-glutamate:ammonia ligase (ADP), EC 6.3.1.2]; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; $\bar{n}$, state of adenylylation (average number of adenylylated subunits per enzyme molecule); EDTA, ethylenediaminetetraacetic acid; MFO, mixed-function oxidation; Tris, tris(hydroxymethyl)-aminomethane.

trum was obtained. Further additions were made to both the reference and sample cuvettes by using Lang–Levy pipets followed by gentle agitation without removing the cuvettes from the instrument.

Dye Binding. The binding of Cibacron Blue to GS was measured by using either differential sedimentation or ultrafiltration techniques. For some of the sedimentation studies, 2.0-mL reaction mixtures containing 10 mM $MgCl_2$, 0.1 M KCl, 10 mM imidazole hydrochloride (pH 7.0), and various amounts of GS (5.8–15.8 μM) and of dye (4–70 μM) were centrifuged at 50 000 rpm for 3 h at 22 °C in a Beckman Model L265B centrifuge equipped with a number 65 rotor. After centrifugation, 1.0 mL of the supernatant solution was carefully withdrawn from the upper portion of the tube. The concentration of free dye in this supernatant fraction was calculated from the absorbance at 610 nm (ϵ = 13.6 mM^{-1} cm^{-1}; Thompson & Stellwagen, 1976). In later studies, 175-μL samples of the dye + GS reaction mixtures were centrifuged at 100 000 rpm for 1 h in a Beckman airfuge. After centrifugation, 50 μL of the supernatant solution was withdrawn for measurement of the free dye concentration.

In control experiments, it was demonstrated that no GS is present in the supernatant fractions following centrifugation and that little or no free dye is sedimented under these conditions. From the difference in absorbance at 610 nm of the samples before and after centrifugation, the amounts of free dye and dye bound to GS could be calculated.

The sedimentation technique could not be used to measure binding of dye to the GS subunit produced in the presence of 4–6 M urea. However, binding of dye to subunits produced by relaxation and dissociation of GS at pH 8.5 could be determined by sedimentation of the GS subunit–dye complex at 100 000 rpm in the airfuge (see above).

The intrinsic dissociation constants of GS–dye complexes formed in the presence of competing ligands (AMP, ADP, and tryptophan) were calculated from measurements of dye binding data obtained in the presence of various concentrations of each ligand, according to the expression

$$K_D = \frac{K_L K_{app}}{[L] + K_L} \quad (1)$$

where K_D = dissociation constant for the GS–dye complex, K_L = dissociation constant of the GS–ligand complex, and K_{app} = apparent dissociation constant of the GS–dye complex as measured in the presence of a given concentration of a competing ligand, [L]. Dye binding in the presence of urea was determined by an ultrafiltration technique using the Amicon micropartition system (MPS-1) fitted with a YMT membrane. One milliliter of the GS–dye reaction mixture was added to the sample reservoir, and the sample was centrifuged for 2–5 min at ~1000g in a table-top centrifuge (Precision Vari-High-Speed Contricone, Model K12 from Precision Scientific Instruments) equipped with a 35° angle rotor. In control experiments, it was demonstrated that due to nonspecific binding of dye to the membrane filter, the absorbance at 610 nm of the first 0.2 mL of filtrate is not a true measure of the unbound dye concentration. Therefore, the first 0.2 mL of filtrate was discarded, and the absorbancy of the next 0.5 mL was used to calculate the amount of free and GS-bound dye concentrations.

Results

Use of Cibacron Blue F_3GA To Detect Conformational Changes Associated with Relaxation and Dissociation of Glutamine Synthetase. As is illustrated in Figure 1, the sequential conversion of taut (native) glutamine synthetase to the relaxed form and then to the dissociated form can be achieved by the addition of 1.0–1.5 mM EDTA, followed by adjustment of the pH to 8.5 (Woolfolk & Stadtman, 1967). Figure 2 depicts the difference spectra obtained when 80 μM dye in the reference cuvette is matched against 80 μM dye + 20 μM enzyme in the sample cuvette, before (curve a) and after (curve b) the addition of 1 mM EDTA at pH 7.4, and then after adjustment of the solutions to pH 8.5 (curve c). Under the conditions of this experiment (i.e., the presence of 0.1 M KCl), the addition of either 1 mM $MnCl_2$ or 1.5 mM EDTA does not affect the spectrum of the dye in the absence of enzyme. Therefore, curves a, b, and c in Figure 2 represent the difference spectra for dye alone vs. dye in the presence of taut, relaxed, and dissociated enzyme, respectively. The difference spectrum obtained with taut enzyme (curve a) exhibits maxima at 600 and 640 nm and a minimum at 700 nm. Relaxation of the enzyme in the presence of EDTA leads to a slight but significant change in the difference spectrum, whereas a marked change occurs upon dissociation of the relaxed enzyme at pH 8.5. Compared to the difference spectrum obtained with taut and relaxed enzyme forms, the difference spectrum obtained with dissociated enzyme (curve c) is greatly red shifted and exhibits a prominent maximum at 650 nm, a shoulder at 610 nm, and a minimum at 712 nm. From direct binding measurements (see Materials and Methods), it will be shown later (Figure 18) that under these conditions the taut and relaxed forms of GS bind 0.9–1.2 mol of dye per mol of enzyme subunit, whereas 2.5–3.0 mol of dye is bound per mol of dissociated subunit.

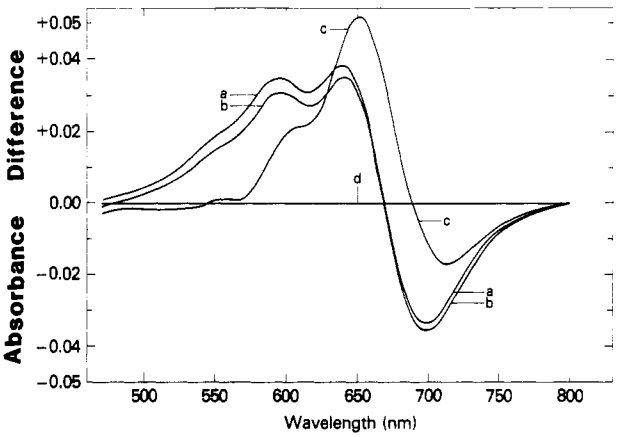

FIGURE 2: Difference spectrum obtained with different structural forms of unadenylylated GS using Cibacron Blue F_3GA. The base line (curve d) was obtained for the standard reaction mixture (80 μM dye, 100 mM KCl, 1 mM $MnCl_2$, and 35 mM Hepes, pH 7.5, ~25 °C) in both the sample and reference cuvettes by using the automatic base-line correction of the Varian 219 spectrophotometer. The difference spectrum for the native taut form of GS (curve a) is produced by the addition of 50 μL of 50–20 mg/mL stock GS solutions to the sample and final dialysate to the reference, respectively. The difference spectra for the relaxed and dissociated forms of GS are produced by the addition of 15 μL of 0.1 M NaEDTA (curve b, relaxed), followed by the addition of 20 μL of a Tris–KOH mixture (see Materials and Methods) to both cuvettes to raise the pH to 8.5.

Although the spectral shift obtained in the presence of EDTA is relatively small, several observations indicate that the change is due to relaxation of the enzyme: (a) If after the addition of EDTA and recording of the difference spectrum (curve b, Figure 2) 1.5 mM $MnCl_2$ is added to both cuvettes, the difference spectrum reverts back to that of the taut enzyme (curve a). This is consistent with the fact that addition of Mn^{2+} to the relaxed enzyme converts it to a tightened configuration that, except for its lower solubility, is indistinguishable from the original taut enzyme (Shapiro & Ginsburg,

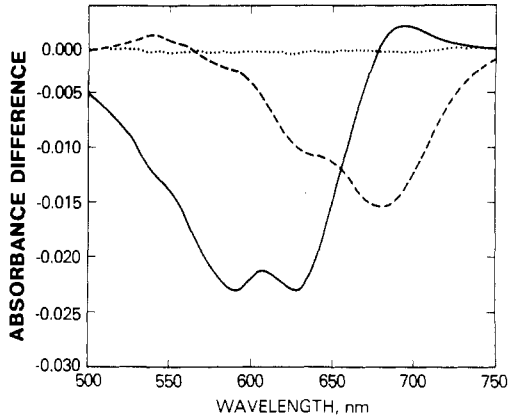

FIGURE 3: Difference spectra between dye bound to relaxed and taut and between dye bound to taut and dissociated forms of GS. Initially, both cuvettes contained 50 mM imidazole hydrochloride (pH 7.0), 1.0 mM $MnCl_2$, 0.1 M KCl, 30 μM dye, and 40 μM GS (subunit equivalents) in a volume of 0.9 mL. After automatic base-line correction, 20 μL of 0.1 M EDTA was added to the sample cuvette, and 20 μL of H_2O was added to the reference cuvette. The difference spectrum of these mixtures (- - -) reflects differences in dye bound to taut and relaxed forms of GS. After the spectrum was recorded, the pH was adjusted to 8.5 by the addition of 16 μL of Tris–KOH reagent (see Materials and Methods) to both cuvettes. The resulting difference spectrum (—) reflects the difference between the spectrum of dye bound to taut GS (reference cuvette) and dye bound to dissociated GS subunits (sample cuvette).

1968). (b) When pure preparations of relaxed enzyme (obtained either by dialysis of EDTA-treated taut enzyme or by passage of taut enzyme through a chelex exchange resin) are added directly to the dye, a difference spectrum identical with curve b (Figure 2) is obtained; however, if either relaxed enzyme preparation is first converted to a tightened configuration by the addition of 1.5 mM $MnCl_2$ (see Figure 1) and then is added to the dye, a difference spectrum identical with that of the taut enzyme (curve a, Figure 2) is obtained (data not shown).

It is noteworthy that conversion of taut to relaxed enzyme elicits a much greater change in the difference spectrum if 0.1 M KCl is omitted from the buffer solution. However, such spectra are more difficult to intepret because, in the absence of KCl, 1.5 mM $MnCl_2$ and 10 mM EDTA have a significant effect on the dye spectrum even in the absence of enzyme. For direct comparison of the spectral differences between dye complexes of taut and relaxed enzyme, an experiment was conducted in which a mixture containing 40 μM (subunit equivalent) taut enzyme and 30 μM dye was added to both the reference and sample cuvettes of the spectrophotometer. After a base-line scan, 15 μL of 0.1 M EDTA was added to the reference cuvette, and 15 μL of water was added to the sample cuvette. At the high enzyme:dye ratio used in this experiment, over 90% of the dye is bound to the enzyme. Therefore, the resulting difference spectrum (Figure 3, dashed line) is almost entirely due to a difference between the taut enzyme–dye and relaxed enzyme–dye complexes. After the difference spectrum of the dye bound to the taut and relaxed enzyme forms (dashed line, Figure 3) was recorded, the reaction mixtures were adjusted to pH 8.5 to promote dissociation of the relaxed but not the taut enzyme. This resulted in a shift in the difference spectrum reflecting a difference in the spectra of dye bound to the taut GS and to its dissociated subunit (Figure 3, solid line).

Comparison of Native GS with GS Inactivated by a Mixed-Function Oxidation System. Any of several mixed-function oxidation systems can catalyze oxidation of GS. This leads to complete loss of catalytic activity. Except for a slight

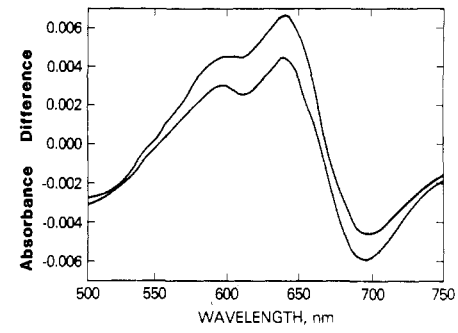

FIGURE 4: Effect of mixed-function oxidation of GS on the dye vs. dye + GS difference spectrum. Initially, both cuvettes contained 60 μM dye, 30 mM Hepes (pH 7.4), and 1.0 mM $MnCl_2$. In addition, the reference cuvette contained 30 μM (subunit equivalents) native $GS_{1.0}$, and the sample cuvette contained 30 μM $GS_{1.0}$ that had been oxidized by the ascorbate, O_2, and $FeCl_3$ mixed-function oxidation system. Spectra were recorded 15 (upper curve) and 45 min (lower curve) after adding GS to the cuvettes.

change in the absorbance at 284–286 nm, the loss of a single histidine residue per subunit, and the appearance of a carbonyl group, the oxidative inactivation of GS produces no significant changes in physicochemical characteristics (Levine et al., 1981; Levine, 1983). Nevertheless, there is a small but significant difference in the spectrum of the dye–protein complexes obtained with native and oxidized enzyme preparations. As shown in Figure 4, the difference spectrum obtained between identical quantities (30 μM) of native and oxidized enzyme in the presence of 60 μM dye exhibits maxima at 644 and 597 nm and minima at 613 and 695 nm. Under the conditions of these measurements, both forms of GS were found to bind 1–1.1 equiv of dye per subunit. Therefore, the difference spectrum (Figure 4) likely reflects differences in the spectrum of the protein–dye complexes. As will be shown in the following paper (Federici & Stadtman, 1985), the commercial preparation of dye used in these experiments is composed of four distinctly different major subspecies. It will be shown that by means of difference spectroscopy as described in Figure 4, one of these subfractions can be used to quantitate the extent of inactivation of GS by the mixed-function oxidation reaction.

Use of Dye-Mediated Difference Spectroscopy To Monitor the Dissociation of GS by Urea. Figure 5 shows the effects of 0.1 M KCl and 1.5 mM EDTA on the time dependence of the dye vs. dye + GS difference spectrum at pH 8.5 in the presence of 2.93 M urea. It is evident from these results that dissociation of GS to subunits is a multiphasic process. This is evident from Figure 5A which shows that in the presence of 0.1 M KCl the difference spectrum obtained immediately after mixing GS with dye exhibits a maximum of low amplitude at 636–640 nm. However, with time, there is a gradual increase in the amplitude of the difference spectrum, a shift in the maximum to 675 nm, and the appearance of a minimum at 575 nm. It is likely that the difference spectrum obtained after prolonged incubation (31 min) is due mainly to binding of dye to monomeric subunits, since a similar spectrum of greater amplitude (dashed line, Figure 5A) is obtained when 1.5 mM EDTA is added to promote complete dissociation of GS. The spectrum obtained immediately after the addition of EDTA did not change with further incubation for 15 min (data not shown). It therefore appears that under these conditions, the relaxed enzyme undergoes complete dissociation almost immediately. From the amplitude of the changes at 575 nm (Figure 5A), it seems likely that about 45% of the subunits were dissociated in 31 min in the absence of EDTA.

Effect of KCl (Ionic Strength). A comparison of the curves in panels A and B of Figure 5 shows that KCl (0.1 M) greatly

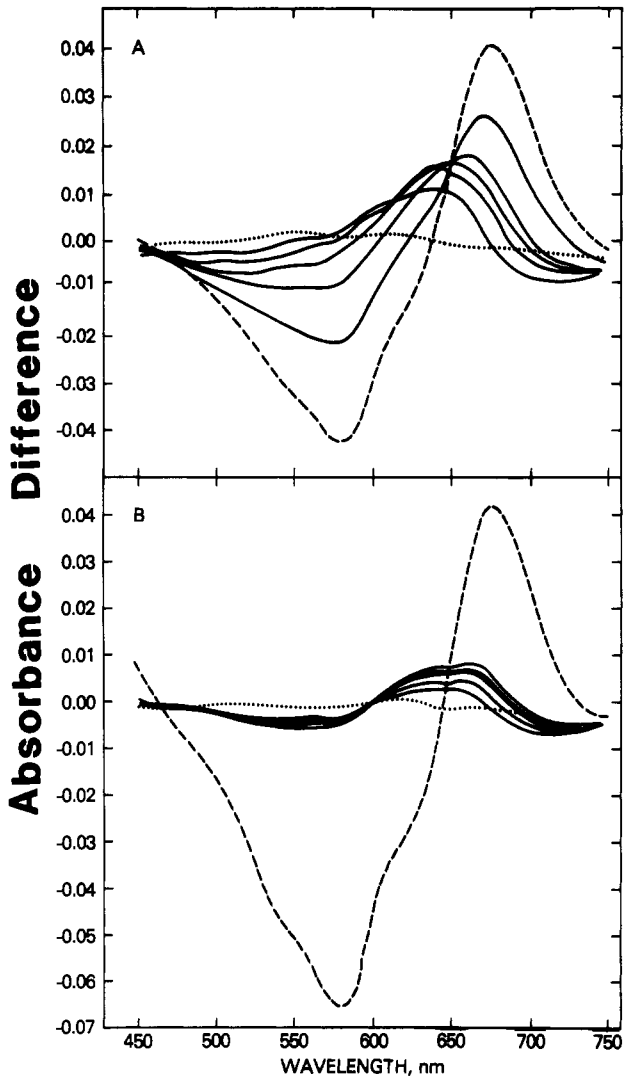

FIGURE 5: Time-dependent changes in the dye vs. dye + GS difference spectrum in the presence of 2.93 M urea. (A) Initially, both cuvettes contained 106 μM dye, 33 mM Hepes (pH 8.5), 0.1 M KCl, and 1 mM $MnCl_2$ (total volume 0.96 mL, 30 °C). At zero time, 40 μL of $GS_{2.3}^{--}$ (21.7 mg/mL) was added to the sample cuvette and 40 μL of buffer to the reference cuvette. Spectra were recorded at 0, 6, 15, and 31 min (solid lines). At 34 min, 1.5 mM EDTA was added (broken lines). (B) Same as (A) except no KCl was present.

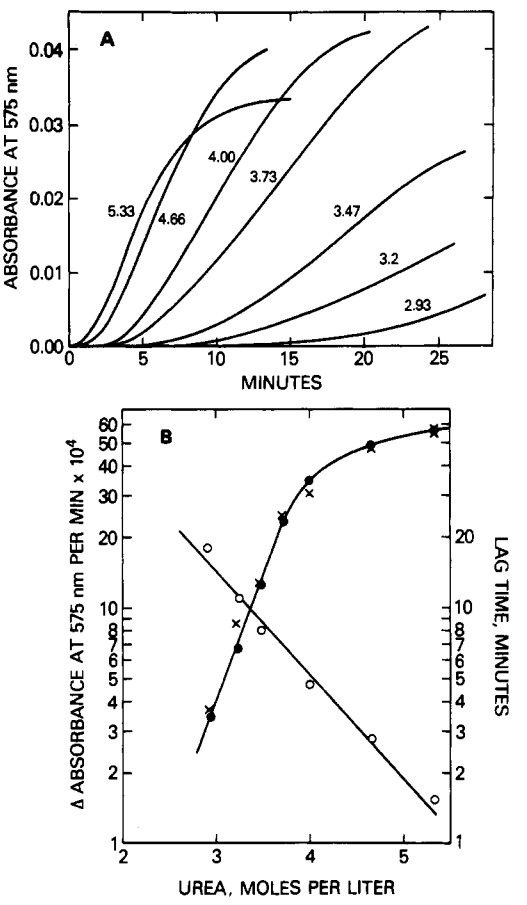

FIGURE 6: (A) Effect of urea concentration on the time-dependent change in spectral differences at 575 nm. Both cuvettes contained 0.1 M KCl, 16.7 mM Hepes (pH 7.5), 106 μM dye, 1.0 mM $MnCl_2$, and molar concentrations of urea as indicated by the numbers on the curves. At zero time, GS to a concentration of 20.4 μM was added to the sample cuvette, and an equivalent amount of buffer was added to the reference cuvette. (B) Effect of urea concentration on the lag time and rate of GS dissociation as monitored at 575 nm. Data are from Figure 6A. The lag (O) times refer to the intercepts on the abscissa obtained by extrapolation of the linear portions of the curves in Figure 6A. The rates (●) are calculated from the slopes of the linear portions of the curves in Figure 6A. (×) refers to results from a similar experiment at pH 7.0. All rate data obtained at pH 7.0 were multiplied by 0.467 to normalize these data with respect to the rates observed with 3.5 M urea at pH 7.5.

facilitates the dissociation of GS in the presence of 1 mM $MnCl_2$ and 2.93 M urea at pH 8.5. In the absence of KCl, little or no dissociation occurred during 30-min incubation (Figure 5B); with addition of EDTA, GS is rapidly dissociated.

The ability of KCl to facilitate dissociation of GS in the presence of urea (no EDTA) is probably related to the affinity of GS for Mn(II). A complex relationship between the affinity of GS for Mn(II) and the concentrations of KCl and urea was disclosed by studies on the stability of GS catalytic activities under various conditions. When $GS_{2.5}^{--}$ was incubated with 0.025 M $MnCl_2$ at pH 8.0 (26 °C), there was no loss of γ-glutamyltransferase activity for up to 45 min, in both the presence and absence of 0.1 M KCl. However, in the presence of 3–4 M urea, over 95% of the transferase activity was lost in 45 min, whether or not 0.1 M KCl was present. The loss of activity could be retarded by increasing the concentration of $MnCl_2$; however, the concentration of Mn(II) required to afford 50% protection was dependent upon the concentrations of both urea and KCl. In the presence of 2.94 M urea, 0.3 and 1.3 mM Mn(II) were required to provide 50% protection of GS from inactivation in the presence and absence of 0.1 M KCl, respectively; with 4.0 M urea, 0.5 and 2.0 mM Mn(II) were needed, respectively. It thus appears that urea decreases the affinity of GS for Mn(II) and that this effect is greatly augmented by the presence of 0.1 M KCl. It follows that KCl and urea together will favor dissociation of the GS–Mn complex and therefore promote conversion of taut GS to the relaxed configuration which undergoes rapid subunit dissociation at high pH (Figure 1).

The ability of urea (2.93 M) to promote dissociation of GS is greatly influenced by the pH. In contrast to the results at pH 8.5 (Figure 5), at pH 8.0 and 0.1 M KCl, little or no dissociation occurs unless EDTA is present. In the presence of EDTA, 0.1 M KCl, and 2.93 M urea, slow dissociation of subunits does occur as is disclosed by the progressive decrease in absorbance at 575 nm and an increase in absorbance at 675 nm over a 15-min period (data not shown).

Kinetics of Urea-Promoted Dissociation. As seen in Figure 5, dissociation of GS in urea can be monitored by measuring the changes in the dye vs. dye + GS difference spectrum which occur at either 575 or 675–700 nm. Factors affecting the kinetics are as follows:

(*a*) *Effect of Urea Concentration.* As shown in Figure 6A, when GS is mixed with dye in the presence of urea (2.93–5.33 M) at pH 7.5 and in the presence of 1.0 mM $MnCl_2$ and 1 M KCl, there is an appreciable lag before any change in absorbance at 575 nm can be detected. This is followed by a nearly linear increase in absorbance with time and finally a period in which the rate of change decreases as dissociation approaches completion. Figure 6B shows that the rate of GS dissociation (i.e., the slope of the linear portions of the change in absorbance vs. time curves in Figure 6A) increases exponentially as the concentration of urea is increased from 2.93 to 4 M, whereas the lag time decreases exponentially over the range of 2.93–5.3 M urea.

Other studies show that the lag time is shortened and the rate of GS dissociation is greatly increased either by the addition of 1.5 mM EDTA or by raising the pH from 7.5 to 8.0. For example, as shown in Figure 6, at pH 7.5 in the presence of 2.9 M urea, GS dissociates at a very slow rate after a lag period of nearly 20 min, whereas with 4 M urea, dissociation occurs after a short lag and is complete after 46 min. By comparison, in the presence of 1.5 mM EDTA, dissociation of GS in 2.9 M urea occurs without any lag and is complete after 26 min; in 4 M urea, dissociation is complete after 6 min. It also appears that the EDTA-promoted dissociation of GS in 2.9 M urea is more complex than in 4 M urea, since in 4 M urea the time-dependent changes in amplitude of the difference spectra are characterized by an isosbestic point at 645 nm. In contrast, the changes observed in 2.9 M urea involve a slight red shift in the maxima at 660–675 nm in addition to an increase in amplitude, and there is no isosbestic point. These differences between the time-dependent changes observed with high and low urea concentrations might reflect different mechanisms of dissociation, or at least differences in the kinetics of individual steps in the dissociation process (see Discussion).

(*b*) *Effect of pH.* The relationship between pH and the rate of GS dissociation in 4 M urea is described by a U-shaped profile. The rate is slowest in the range of pH 7.7–8.2 but increases rapidly (about 2.6-fold) as the pH is either increased to 8.5 or decreased to 7.0. The effect of pH on the rate of dissociation appears to be independent of the urea concentration over the range of 2.93–5.3 M. Thus, when the data obtained at pH 7.0 and 7.5 were normalized with respect to the rates observed in 3.5 M urea, the relationship between the concentration of urea and the rate of dissociation at pH 7.0 is identical with that observed at pH 7.5.

(*c*) *Effect of Temperature on Urea-Promoted Dissociation of GS.* Immediately after mixing GS with dye in sample cuvettes containing 4.8 M urea and 0.1 M KCl at pH 8.0, the time course of absorbance changes at 580, 638, and 700 nm was followed at 10, 20, and 31 °C. The multiphasic nature of urea-promoted dissociation of GS is evident from the results shown in Figure 7. At all three temperatures, there was an almost instantaneous increase, to about the same level, in the absorbance difference (dye vs. dye + GS) at 638 nm (Figure 7, open circles), reflecting the primary interaction of dye with GS and probably partial dissociation of GS to aggregates containing two or more subunits. The absorbance difference at 638 nm remained essentially stationary for several minutes and then decreased to the base-line level. With increasing temperature, the length of the stationary phase decreased, and the rate of decline to the base-line value increased. Immediately after addition of GS to the dye, there was little or no absorbance difference at 580 or 700 nm, but after a lag, toward the end of the stationary phase monitored at 638 nm, the

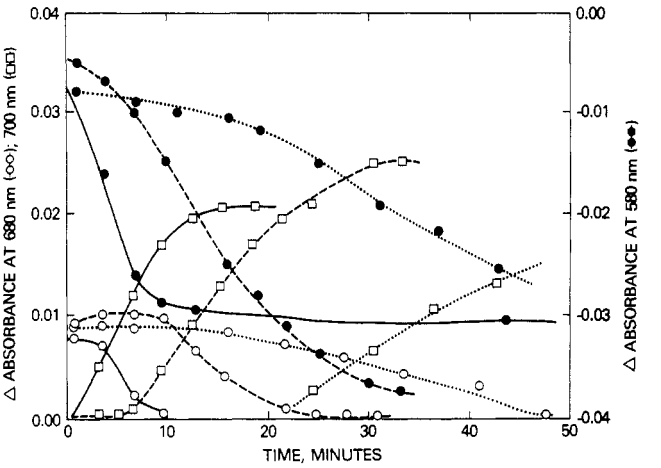

FIGURE 7: Effect of temperature on the kinetics of urea-promoted GS dissociation as monitored by changes in the dye vs. dye + GS spectrum at 580, 638, and 700 nm. Initially, both cuvettes contained 4.78 M urea, 109 μM dye, 33 mM Hepes (pH 8.0), 0.1 M KCl, and 40 μM $MnCl_2$. In addition, the sample cuvette contained 0.85 mg of $GS_{\overline{2.3}}$. The amplitudes of the spectral changes at 580 (●), 638 (○), and 700 nm (□) were measured as a function of time at 10 (···), 20 (- - -), and 31 °C (—).

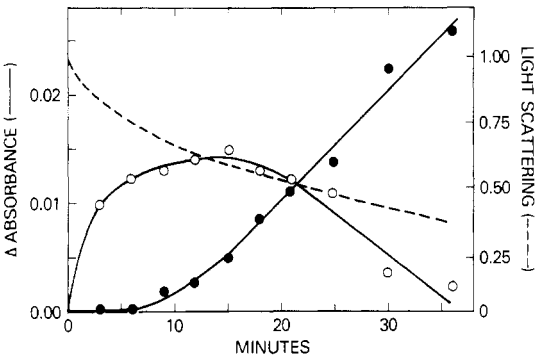

FIGURE 8: Relationship between changes in the light-scatter ratio and the changes in the dye vs. dye + GS difference spectrum caused by urea-promoted dissociation of GS. Changes in the dye vs. dye + GS spectra were measured at 635 (○) and 575 nm (●) under the conditions described in Figure 7. The light-scattering measurements were measured under identical incubation conditions using a Hitachi-Perkin-Elmer MPF-2A spectrofluorometer.

absorbance at 580 nm began to decrease (Figure 7, closed circles) and that at 700 nm (Figure 7, open squares) began to increase at rates that increase with increasing temperature. It is during this latter phase that GS is presumed to undergo dissociation to monomeric subunits. To verify this conclusion, a similar experiment was carried out in the presence of 2.93 M urea and 0.1 M KCl at pH 8.5. In addition to monitoring the changes in absorbance at 635 and 575 nm, the change in light scattering was also monitored (see Materials and Methods). As shown in Figure 8, the rapid increase in absorbance at 635 nm which occurs upon mixing GS with dye is associated with a rapid decrease in light scattering which assumes a linear rate of decline at about the same time that the absorbance at 575 nm assumes a linear rate of decrease. Note also that the change in light scattering and the absorbance at 575 nm continue to change at constant rates even after the absorbance at 638 nm declined almost to the base-line level. The system is obviously too complex to allow definitive conclusions; nevertheless, the results illustrate the ability of difference spectroscopy to monitor changes in the state of aggregation of GS subunits. We believe that the rapid decrease in light scattering that accompanies the increase in

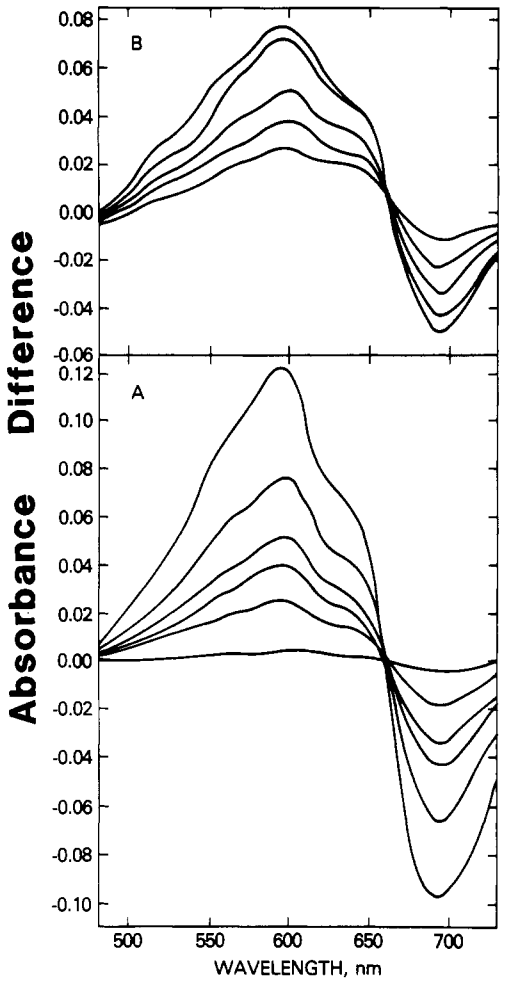

FIGURE 9: Effect of dye concentration on stacking. (A) A concentrated solution of dye (in H_2O) was placed in a 0.5-cm cuvette in the reference compartment, and a 1:10 dilution of the dye solution was placed in a 5.0-cm cuvette in the sample compartment. In descending order, with respect to absorbancy at 586 nm, the curves represent difference spectra obtained with 152, 105, 76, 62, 42, and 17 μM dye in the 0.5-cm cuvette. (B) The absolute spectra of solutions containing 5, 10, 20, 30, 50, and 60 μM dye were measured, stored on a magnetic tape, and analyzed with a HP85 microcomputer. Each spectrum (except that for 60 μM dye) was multiplied by the factor 60/dye concentration and was subtracted from the spectrum obtained with 60 μM dye. In descending order with respect to absorbancy at 586 nm, the curves represent the calculated difference spectra for data obtained with 5, 10, 20, 30, and 50 μM dye solutions.

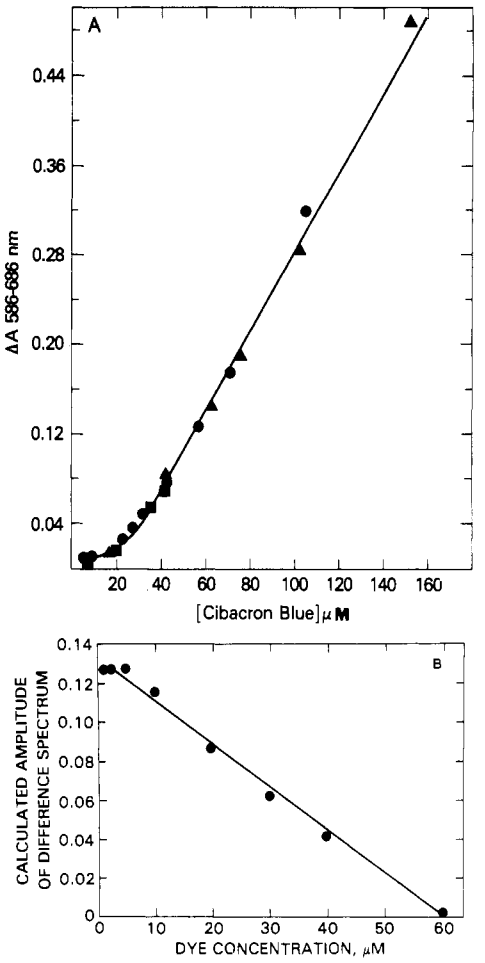

FIGURE 10: Relationship between the amplitude of the difference spectra due to stacking and the concentration of dye. (A) The amplitudes of the absorbance differences between 586 and 686 nm were calculated from the curves in Figure 14A (closed triangles) and from other similar experiments (closed circles and closed squares) (data not shown). (B) Amplitudes of the calculated absorbance differences derived from the data in Figure 14B.

absorbance at 638 nm is attributable to dissociation of the GS to aggregates of intermediate size (Woolfolk & Stadtman, 1967; Whitely & Ginsburg, 1978; Maurizi & Ginsburg, 1982) and that the change at 575 nm is attributable to the formation of monomers which are known from other studies to be formed under these conditions (Ciardi et al., 1973; Woolfolk & Stadtman, 1967a,b), and which upon interaction with dye give rise to a difference spectrum exhibiting a minimum at 575 nm (cf. Figure 5).

Dye Stacking. In the course of these studies, a number of observations indicated that at least part of the dye vs. dye + GS difference spectrum might be attributed to shifts in the equilibrium between stacked and unstacked dye molecules. Such stacking is a common property of aromatic compounds and has been shown to affect their absorption spectrum (Rabinowitch & Epstein, 1942; Schnabel et al., 1962; Davidson, 1972). The possibility that dye stacking might be involved in the present studies was suggested by (a) discrepancies between stoichiometry of dye binding to GS as deduced from difference spectroscopy and from direct binding measurements, (b) variations in dissociation constants of the dye–GS complex as calculated from spectral measurements, and (c) anomalous behavior of the interaction of dye with Lubrol WX. The fact that Cibacron Blue is partially stacked under our experimental conditions and that the stacking does provoke a significant shift in the absorption spectrum was directly demonstrated by two different techniques. In the first technique, a sample of dye was placed in a cuvette having a 0.5-cm light path in the reference compartment, and a 1:10 dilution of the dye was placed in a cuvette having a 5-cm light path in the sample compartment. In the absence of dye stacking, the absorbance of the solution in both cells should be identical. The generation of a difference spectrum under these conditions is therefore evidence for the existence of a concentration-dependent reaction (i.e., stacking). Figure 9A shows the difference spectra generated in a series of such experiments in which the concentration of dye in the 0.5-cm cells was varied from 16.8 to 150 μM, with concentrations in the 5-cm cells set at 0.1 these values. It can be seen that a family of curves are generated that exhibit a maximum at 586 nm and a minimum at 686 nm. The possibility that the observed difference spectra are artifacts caused by imperfect alignment of the cuvettes or by unknown factors was discounted by the demonstration that no difference spectrum was obtained when the colored nonaromatic Co(III) complex $[Co(en)_2CO_3]Cl$, which also absorbs light at 550–700 nm, was

substituted for the dye. Figure 10A (closed circles and closed triangles) shows that the amplitude of the difference spectrum between the concentrated and 1:10 diluted dye solutions in 0.5- and 5.0-cm cells, respectively, is a linear function of dye concentration (0.5-cm cell) over the range 20–150 μM. Results almost identical with those shown in Figures 9A and 10A were obtained by a slightly different technique. In this case, the absolute spectra of a concentrated dye solution and of an appropriate dilution thereof were measured with a Hewlett-Packard 8450A spectrophotometer or with a Varian 219 spectrophotometer interfaced with a Hewlett-Packard HP-85 microcomputer (programmed by R. L. Levine). Each of the absolute spectra was stored in the memory, and the difference spectrum was generated by multiplying the absolute spectrum of each diluted sample by an appropriate factor and subtracting from it the spectrum of the corresponding concentrated sample. The difference spectra generated in this manner are quanitatively and quantitatively identical with those generated by the above procedure (cf. Figure 9A,B). Figure 10B illustrates the relationship between the amplitude of the calculated difference spectrum and the dye concentration for a series in which the absolute absorbance of a 60 μM dye solution is subtracted from the calculated spectrum obtained when the absolute spectrum of a more dilute dye solution (2–50 μM) is multiplied by the dilution factor, 60/dye concentration. It is noteworthy that the relationship is linear over the range of 5–50 μM dye and is discontinuous at concentrations below 5 μM. This suggests that at concentrations below 5 μM, the dye exists mainly as unstacked molecules.

Effect of Solvent on Dye Stacking. A number of factors are known to affect the stacking of aromatic compounds (viz., detergents, ionic strength, and dielectric constant). The effects of urea, Lubrol, dioxane, and KCl concentrations on the degree of Cibacron Blue stacking are illustrated in Figure 11. As the concentrations of Lubrol, urea, and dioxane were increased, the amplitude of the absorbance difference between 100 μM dye in a 0.5-cm cell and 10 μM dye in a 5-cm cell decreased, and with high concentrations of any one of these agents, the difference spectrum was almost abolished. It thus appears that high concentrations of these reagents can cause complete conversion of stacked molecules to monomers. If so, spectral perturbations similar to those shown in Figure 11 should be generated when solutions of dye alone are matched against the same solution of dye supplemented with either Lubrol or urea. This is in fact the case. The difference spectra obtained for dye vs. dye + 4 M urea and for dye vs. dye + 0.04% Lubrol WX [poly(oxyethylene) alcohol] are very similar to the spectra in panels A and B, respectively, of Figure 11 (data not shown). Other surfactant agents, Triton and Cremophor EL, produce spectral perturbations similar to that produced by Lubrol. Although spectral differences generated by any one of these reagents are probably due in part to destacking of the dye, other factors must be involved since each reagent elicits a different spectral perturbation. As noted by others (Thompson & Stellwagen, 1976; Subramanian & Kaufman, 1980), the hydrophobicity and ionic strength of the solvent may have a direct effect on the dye spectrum.

Effect of Lubrol Concentration on the Spectrum of Cibacron Blue. As shown in Figure 12, the amplitude of the difference spectrum between 38 μM dye vs. 38 μM dye + Lubrol increases as the concentration of Lubrol is increased over the range of 0.1–4 mM. When the amplitudes of the difference spectra are plotted against the log of the detergent concentration as described by Shinoda et al. (1963), a sigmoidal-shaped curve is obtained which is presumed to reflect

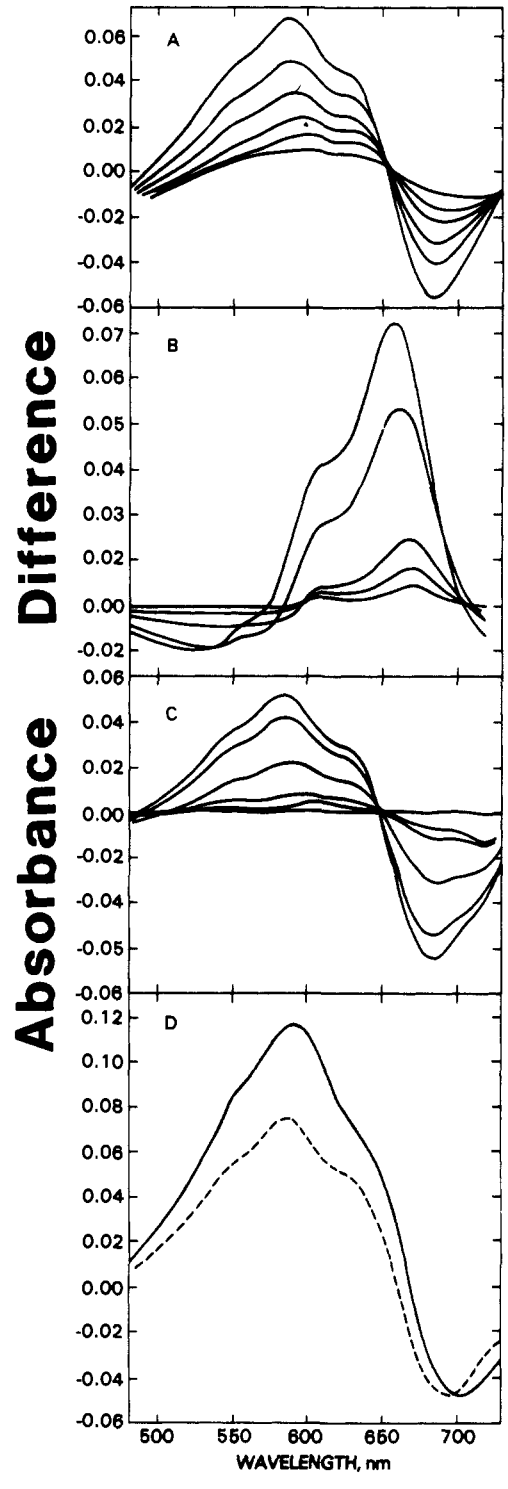

FIGURE 11: Effect of urea, Lubrol, dioxane, and KCl on the difference spectrum between solutions of stacked and unstacked Cibacron Blue dye. Each curve is the difference spectrum obtained when a 100 μM dye solution in a cuvette with a 0.5-cm light path (reference cuvette) is matched against a 10 μM solution of the dye in a 5.0-cm light path (sample cuvette). (A) The curves identified in descending order at 575 nm represent spectra obtained in the presence of 0, 1, 2, 3, 4, and 6 M urea. (B) In descending order with respect to the absorbance at 660 nm, the curves represent difference spectra obtained in the presence of 0.05, 0.1, 0.2, 0.3, and 0.4% Lubrol. (C) In descending order with respect to the peak absorbance at 575 nm, the curves represent difference spectra obtained in the presence of 1, 2, 5, 10, 15, and 75% v/v of dioxane. (D) The solid and dashed lines depict the spectra obtained in the presence of 0.5 and 0.24 M KCl, respectively.

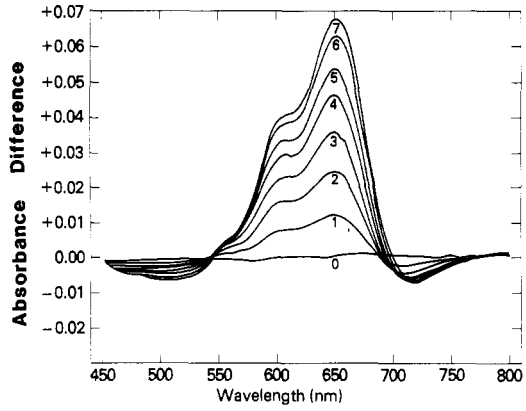

FIGURE 12: Titration of Cibacron Blue F_3GA with increasing concentrations of Lubrol WX. The base line (curve 0) was obtained with 38 μM dye and 20 mM Tris (pH 7.53) in both the sample and reference cuvettes. Sequential additions of 1.83% Lubrol WX were made to the sample compartment with buffer added to the reference generating the observed difference spectra (1–6).

a partition equilibrium between the micelles and the bulk of the solution; when more micelles form, more dye is "bound" to the micelles (Carey & Small, 1969). The above authors have used the point of zero difference absorbance in a dye spectrum as the critical micelle concentration of the detergent. Extrapolation of our data to the point of zero difference absorbance gives a value of 85 μM which is in good agreement with the reported critical micelle concentration of Lubrol WX (Tanford & Reynolds, 1976; Helenius & Simons, 1975).

Effects of Salts. In sharp contrast to the effects of urea, Lubrol, and dioxane, high concentrations of salt increase the extent of dye stacking. Thus, as shown in Figure 11D, as the concentration of KCl was increased from 0.24 to 0.5 M, the amplitude of the spectral difference between 100 μM dye in a 0.5-cm cuvette and 10 μM dye in a 5.0-cm cell increased. As predicted from this behavior, when a given concentration of dye in the absence of KCl is matched against the same concentration of dye in the presence of KCl (both in 1.0-cm cells), the resulting difference spectrum (Figure 13B) is qualitatively the same as that produced by stacking (Figure 9).[2] Furthermore, the amplitude of the difference spectrum thus obtained is nearly proportional to the concentration of KCl over the range of 0.1–0.8 M (inset, Figure 13B).

Figure 13A shows that nearly identical spectral perturbations are produced with other salts, including sodium acetate, arginine hydrochloride, and lysine hydrochloride, whereas equal concentrations of the neutral amino acid glycine (also alanine; see Figure 15) and the dipeptide glycylglycine have little effect on the dye spectrum.

Effect of Salts on GS–Dye vs. Dye Difference Spectrum. The presence of high salt concentrations also affects the difference spectrum between GS + dye and dye alone. As shown in Figure 14, the difference spectrum obtained in the presence of buffer alone has a maximum at 645 nm and a shoulder at ca. 600 nm; however, in the presence of 0.1 M KCl, arginine hydrochloride, lysine hydrochloride, or 25 mM tryptophan, the spectrum is shifted to one exhibiting maxima at 630 and 595 nm.

The effect of tryptophan is particularly noteworthy since this is a neutral amino acid and should not contribute to the ionic strength (cf. results with glycine and alanine; Figures 13A and 15) of the medium. The effect of tryptophan is likely due in part to its interaction with the dye (stacking interaction) and also because it competes with dye in the binding to GS. Evidence that tryptophan interacts directly with the dye is

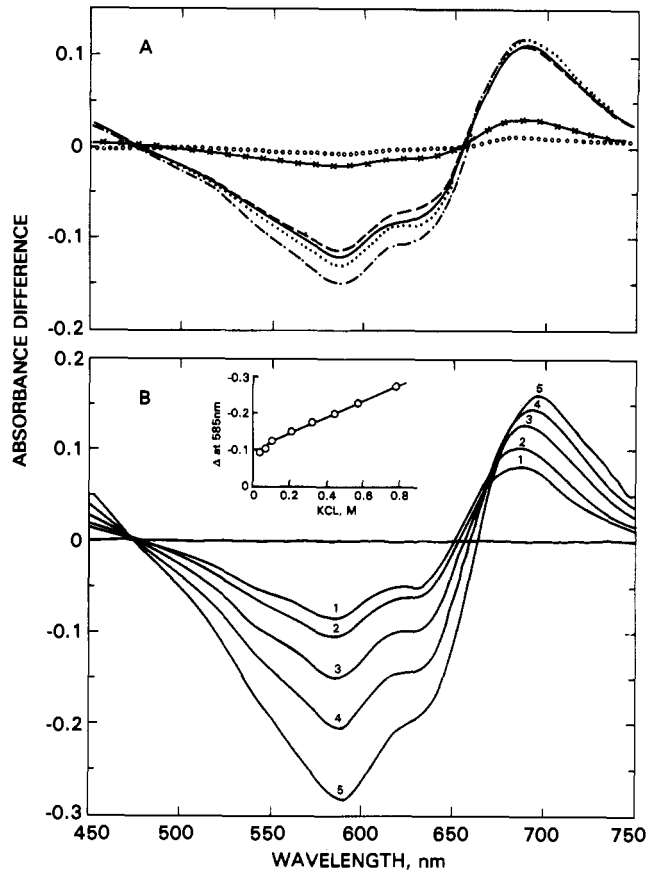

FIGURE 13: Effect of salts on the spectrum of Cibacron Blue. Each cuvette contained 60 μM dye. (A) The sample cuvette contained 0.1 M either glycine, (O), glycylglycine (×), sodium acetate (- - -), KCl (—), arginine hydrochloride (···), or lysine hydrochloride (–·–). (B) Curves 1, 2, 3, 4, and 5 are difference spectra obtained when 26.6, 52.6, 200, 451, and 780 mM KCl, respectively, were present in the sample cuvette. The insert shows the relationship between the amplitude of the change in absorbance at 585 nm and the concentration of KCl in the sample cuvette.

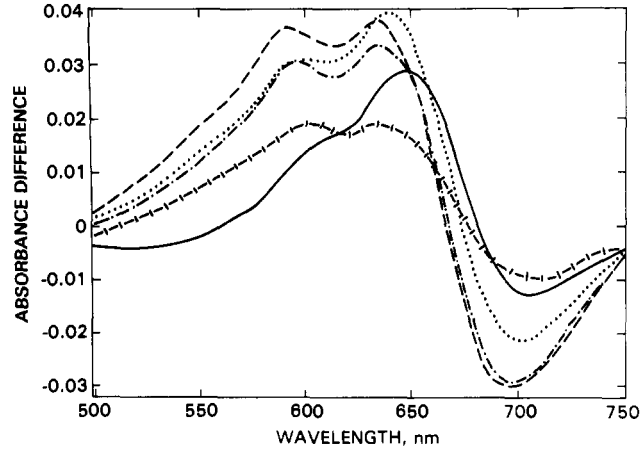

FIGURE 14: Effect of salts on the difference spectrum between dye and the GS–dye complex. Each cuvette contained 60 μM dye, 33 μM Hepes buffer (pH 7.4), and 1.0 mM $MnCl_2$. As indicated by the symbols, lysine hydrochloride (- - -), KCl (···), and arginine hydrochloride (–·–) were present at 0.1 M; tryptophan (|–|) was present at 24.5 mM; no added salt (—). The sample cuvette contained 20.6 μM GS (subunit equivalent).

[2] In the experiment of Figure 9, the cuvette containing the highest concentration of dye (0.5-cm cell) was in the reference compartment, whereas in the experiment of Figure 13B, it was in the sample compartment (high salt concentration). Therefore, in comparison of the spectra of Figures 9 and 13B, one set of spectra must be inverted.

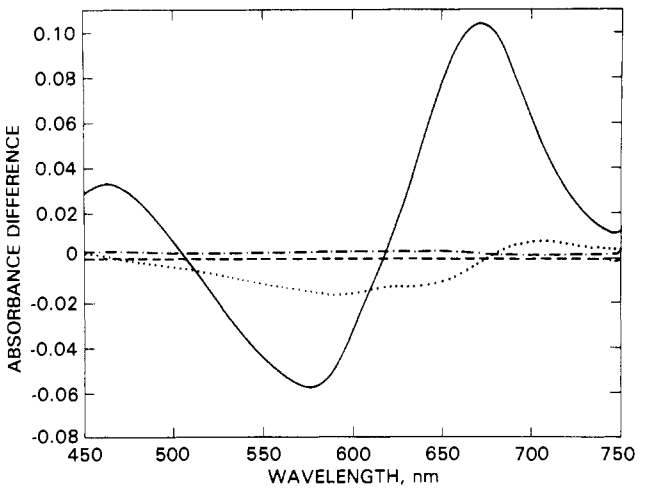

FIGURE 15: Effects of tryptophan and alanine on the dye spectrum. Each cuvette contained 60 μM dye, 33 mM Hepes (pH 7.4), and 0.1 M KCl. In one case (—), the sample cuvette contained 41 μM tryptophan; in another case (-·-), the sample cuvette contained 41 μM alanine; in a third case (···), the sample cuvette contained 41 μM additional KCl. The dashed line (---) represents the base line.

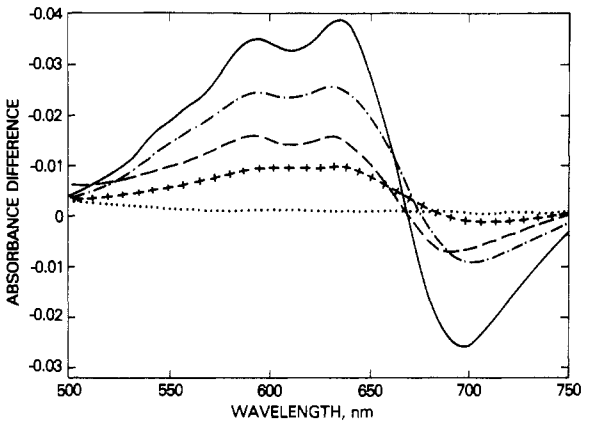

FIGURE 16: Effect of competing ligands, tryptophan, and ADP on the amplitude of the dye vs. dye + GS difference spectrum in 0.2 M KCl. Each cuvette contained 38.2 μM dye, 0.2 M KCl, 3 mM $MnCl_2$, 33 mM Hepes (pH 7.4), and either no ligand (—), 24.5 μM tryptophan (-·-), 142 μM ADP (---), 214 μM ADP (+), or 2868 μM ADP (···). The sample cuvette contained 20.6 μM GS (subunit equivalents).

presented in Figure 15. This shows the difference spectrum of dye + 41 μM tryptophan vs. dye alone. The rather large amplitude of the difference spectrum due to tryptophan cannot be due to an ionic strength effect since as already noted tryptophan is a neutral amino acid; moreover, the experiment was carried out in the presence of 0.1 M KCl. Further addition of 41 mM KCl produces only a minor change in the difference spectrum compared to that produced by 41 mM tryptophan (Figure 15). The inability of other neutral amino acids to produce a similar effect is shown by the fact that the addition of 41 mM alanine on top of 0.1 M KCl has no significant effect on the dye spectrum (Figure 15). These results indicate that perturbation of the dye spectrum is obtained by the direct interaction of the dye with tryptophan.

Ability of Tryptophan, ADP, and AMP To Compete with Cibacron Blue Binding. Because the binding of Cibacron Blue to enzymes is often quenched by the addition of nucleotides, it has been concluded that the dye binds to the nucleotide binding site on the enzyme. This appears to be true also in the case of GS. As shown in Figure 16, the amplitude of the dye vs. dye + GS difference spectrum at 675 nm (in the presence of 0.2 M KCl) is progressively decreased by adding increasing amounts of ADP, over the range of 0.14–2.9 mM.

Table I: Determination of the Dissociation Constant of the $GS_{\overline{1.0}}$–Dye Complex from Direct Binding Measurements

composition of reaction mixtures[a]					
$[dye]_t$ (μM)	$[GS]_t$ (μM)	$[dye]_f$ (μM)	[dye–GS] (μM)	$[GS]_f$ (μM)	K_D (μM)
9.60	10.40	2.14	7.46	2.94	0.84
8.64	9.36	1.99	6.65	2.71	0.81
6.72	7.28	1.58	5.14	2.14	0.66
4.80	5.20	1.26	3.54	1.66	0.59
3.80	4.16	1.10	2.74	1.42	0.57
2.88	3.12	0.80	2.08	1.04	0.40
8.91	11.6	1.13	7.78	3.83	0.56
7.13	11.6	0.48	6.65	4.95	0.34
8.91	5.8	3.86	5.05	0.75	0.59
7.13	5.8	1.88	5.25	0.55	0.20

[a] In addition to the concentration of $GS_{\overline{1.0}}$ and dye as indicated, each 2.0-mL reaction mixture contained 10 mM $MgCl_2$, 10 mM imidazole hydrochloride buffer (pH 7.0), and 100 mM KCl. Samples were centrifuged for 3 h at 50 000 rpm, 25 °C. The concentration of the free dye ($[dye]_f$) in the supernatant was calculated from the absorbances at 610 nm. The concentration of the GS–dye complex is assumed to be equal to the difference between the total and free dye concentrations (i.e., [GS–dye] = $[dye]_t - [dye]_f$); the concentration of free GS is assumed to be equal to the difference between the total GS concentration and the GS–dye complex concentration (i.e., $[GS]_f = [GS]_t -$ [GS–dye]).

Further evidence that this effect is due to competition for binding at the catalytic site was obtained by studying the ability of Cibacron Blue to inhibit the ADP-dependent γ-glutamyltransferase activity of GS. Plots of $1/v$ vs. the dye concentration at three different levels of ADP yielded straight lines which intersect at a common point above the abscissa and to the left of the ordinate; plots of s/v vs. dye concentration yield a series of parallel lines. These results indicate that the dye is a competitive inhibitor of GS with respect to ADP (K_i = 0.85 μM). Results of other studies (P. Z. Smyrniotis and E. R. Stadtman, unpublished results) show that the allosteric effectors AMP and tryptophan decrease the affinity of GS for ADP. It is therefore not surprising that these ligands affect also the binding of Cibacron Blue to the enzyme. The ability of tryptophan to quench the dye vs. dye + GS difference spectrum is shown in Figure 16; AMP has a similar effect (data not shown).

The competitive effect of these ligands is quantitatively described by increases in the apparent dissociation constants of the GS complexes, as shown in Table II, column 3. Since the dissociation constants of the complexes between GS and the competing ligands are known (Whitley & Ginsburg, 1978), it is possible with eq 1 (see Materials and Methods) to calculate the intrinsic binding constant of the GS–dye complex from the data in Table I, columns 2–4. As shown in column 6 of Table I, the calculated K_D values are in good agreement with an average value of 0.55 μM which was determined by direct measurements in the absence of competing ligands (Table I). Similarly, with eq 1 and a value of 0.55 μM for K_D, the dissociation constants of the other GS–ligand complexes can be calculated from the data in Table II, columns 2 and 3. There is good agreement between the calculated values (column 5, Table II) and the experimentally determined values (column 4, Table II).

Relationship between GS and Dye Concentration on the Difference Spectrum. Figure 17 shows how the amplitude of the difference spectrum varied when 20 μM concentrations of the taut and dissociated forms of GS were titrated with increasing concentrations of dye over the range of 10–100 μM. On the basis of a value of 0.55 μM for the dissociation constant of the dye–GS complex (Table I), as well as the data in Figure

Table II: Dissociation Constants of GS–Ligand Complexes Derived from Dye Binding Data[a]

ligand added	concn (μM)	K_{app}[b] (μM)	K_L(calcd)[d] (μM)	K_L(obsd)[c] (μM)	K_D[e] (μM)
tryptophan	7500	4.3	1100	910	0.47
tryptophan	15000	7.2	1240	910	0.41
ADP	10	3.0	2.24	3	0.69
ADP	20	4.9	2.47	3	0.64
AMP	1000	5.1	118	130	0.59

[a] Reaction mixtures contained 11.4 μM GS (subunit equivalents), 10.5 μM Cibacron Blue F_3GA, 10 mM imidazole hydrochloride buffer (pH 7.0), 100 mM KCl, and ligand as indicated. The value of K_{app} was calculated from the concentration of unbound dye as determined by the sedimentation technique described under Materials and Methods. [b] K_{app} = apparent dissociation constant of the GS–dye complex calculated from binding data obtained in the presence of competing ligands, as indicated. [c] K_L(obsd) = dissociation constant of the GS–ligand complex determined by direct binding measurements [see Whitley & Ginsburg (1978)]. [d] K_L(calcd) = dissociation constant of the GS–ligand complex calculated according to eq 1 by using the data in columns 2 and 3 and a value of 0.55 μM for K_D (i.e., an average of all values in Table I). [e] K_D = intrinsic dissociation constant of the GS–dye complex calculated from the data in columns 2 and 3 (this table) by using eq 1 and previously determined dissociation constants for the GS–ligands as indicated in column 5.

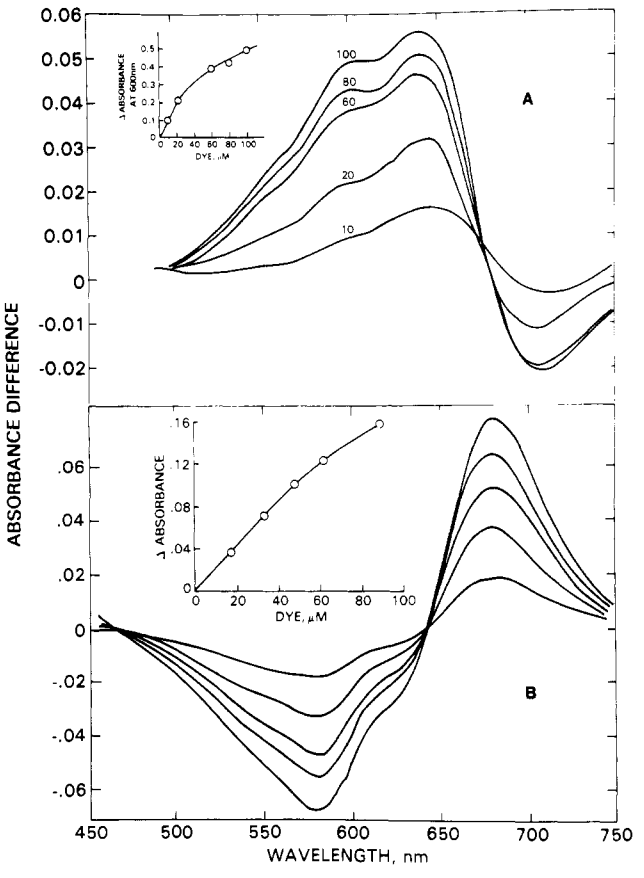

FIGURE 17: Effect of dye concentration on the amplitude of the dye + GS vs. dye difference spectrum. (A) Titration of taut enzyme. Both cuvettes contained 50 mM Hepes buffer (pH 7.4), 3.0 mM $MnCl_2$, 0.3 M KCl, and the concentration (in micromolar) of dye as indicated by the numbers on the curves, 30 °C. In addition, the sample cuvette contained 20 μM GS (subunit equivalents). (B) Titration of dissociated subunits in 4 M urea. Initially, both cuvettes contained 4 M urea, 20 mM Tris (pH 7.5), and 3.3 mM EDTA. In addition, the sample cuvette contained 20 μM $GS_{2,3}$. Both cuvettes were then titrated with increasing amounts of dye (1600 μM). Final concentrations of dye for curves from bottom to top (at 535 nm) were 16.9, 32.4, 47.1, 61.4, and 88 μM.

18, the taut form of GS should be almost completely saturated with dye at dye concentrations of 60 μM. Yet, as shown by

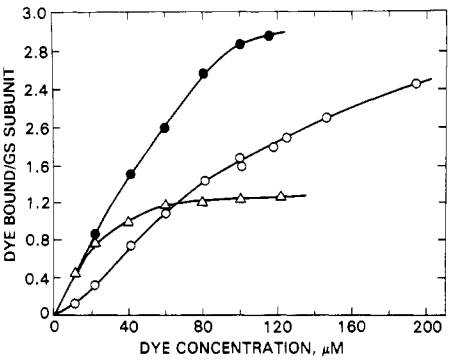

FIGURE 18: Binding of dye to taut and dissociated forms of GS. Incubation mixtures contained 30 mM Tris (pH 7.5), 45 μM $MnCl_2$, 4.6 mM KCl, 22.6 μM GS (subunit equivalents), and other additions as follows: (Δ) no additions (i.e., taut enzyme); (○) 3.3 mM EDTA, pH adjusted to 8.7 (i.e., dissociated enzyme); (●) 3.3 M EDTA and 4 M urea (i.e., dissociated enzyme, pH 7.5). After 60 min, the amount of dye bound per subunit of GS was determined by the ultrafiltration technique as described under Materials and Methods.

the inset in Figure 17A, the amplitude of the difference spectrum continues to increase with increasing dye concentrations over the range of 60–90 μM. This phenomenon is likely related to dye stacking. Whereas much of the unbound dye exists in a stacked state, only the monomolecular form appears to be bound to the taut form of GS. Otherwise, the number of dye molecules capable of binding to taut GS would greatly exceed the number of GS subunits. This is clearly not the case (Table I, Figure 18). In an experiment such as that described in Figure 17A, it can be calculated that when the concentration of dye is equal to the GS subunit concentration, 80–95% of the dye in the GS + dye compartment will be bound to GS in an unstacked state. This dye–GS complex will be in equilibrium with a relatively low concentration of free dye, which, because of its low concentration, will also be present mostly as unstacked dye. In other words, with equal concentrations of dye and GS, the difference spectrum between dye vs. dye + 20 μM GS subunits reflects largely the difference spectrum between stacked and unstacked dye molecules. However, as the concentration of dye in the two compartments is increased further, the amount of stacked dye in the reference cell containing dye alone will increase more rapidly than that in the sample cell containing dye + GS, because the concentration of free (unbound) dye in the sample cell will always be 20 μM lower than that in the reference cell. Hence, as the total concentration of dye is increased above 20 μM, the amount of stacked dye in the reference cell will continue to increase relative to the amount of stacked dye in the sample cell, and the amount of unstacked dye in the reference cell will continue to decrease relative to the total unstacked dye (i.e., free dye monomer + dye–GS complex) in the sample cell. Thus, the amplitude of the difference spectrum, part of which reflects the net difference in concentrations of stacked and unstacked dye in the two cell compartments, will continue to increase, even after the GS is saturated with dye. This interpretation is supported by a theoretical analysis, the results of which are shown in Figure 19. For simplicity, it was assumed that dye stacking leads to the formation of dimers and that only one monomeric dye molecule can bind to each subunit of GS. It was also assumed that the total concentration of unstacked dye in the presence of GS is equal to the sum of the concentrations of free monomers and the dye–GS complex. With these assumptions, the relative concentrations of unstacked dye in the presence and absence of GS were calculated as a function of dye concentration; for these calculations, the dissociation constant of stacked dye (dimer) was

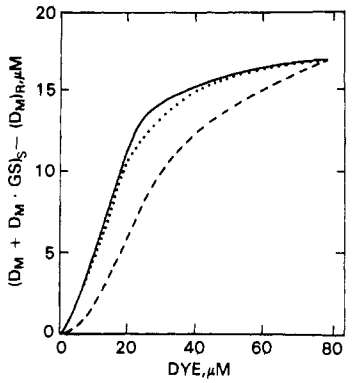

FIGURE 19: Effect of dye concentration on the calculated distribution of unstacked dye molecules in the presence and absence of GS. Computer simulation of the relationship between dye concentration and the concentration of unstacked dye (D_M) in the presence and absence of 20 μM GS subunits. Dissociation constants for stacked dye (K_S) and for the dye–GS complex (K_{DG}) were assumed as follows: (—) K_S = 50 μM, K_D = 1.0 μM; (- - -) K_S = 5 μM, K_D = 0.1 μM; (···) K_S = 5 μM, K_D = 1 μM. The ordinate = $(D_M + D_M\text{–GS})_S - (D_M)_R$, where S and R denote the concentration in sample and reference compartments, respectively. With the assumption (see text) that D_M and D_M–GS have identical spectra, it follows that the ordinate, $(D_M + D_M\text{–GS})_S - (D_M)_R$, represents the excess of dye monomers in the sample cuvette over that in the reference cuvette. Because $(D_M + D_M\text{–GS})_S - (D_M)_R = (D_S)_R - (D_S)_S$, where $(D_S)_R$ and $(D_S)_S$ represent the concentration of stacked dye molecules in the reference and sample cuvettes, respectively, it follows that if the extent to which the level of dye monomers in the sample cuvette exceeds the level of monomers in the reference cuvette, there will be a corresponding increase in the level of stacked dye in the reference cuvette compared to that in the sample cuvette. It is noteworthy that only the excess of a given species of dye in one cuvette over that in the other can contribute to the difference spectrum. Consequently, as the ordinate value of $(D_M + D_M\text{–GS})_S - (D_M)_R$ increases, there will be an increase in the amplitude of the difference spectrum which reflects the difference in absorbance of the stacked and unstacked dye.

assumed to be either 5 or 50 μM, and the dissociation constant of the dye–GS complex was assumed to be either 0.1 or 1.0 μM. As shown in Figure 19, for each of these theoretical situations examined, the difference between the amounts of unstacked dye in the presence and absence of GS continues to increase, even after 1 equiv of dye is bound to each subunit of GS. It therefore follows that if the absorption spectra of free monomer and of dye bound to monomer are the same, then the difference spectrum between dye and dye + GS will increase with increasing dye concentrations well beyond the point when GS is saturated with dye.

Difficulties in measuring the stoichiometry of the dye binding by difference spectroscopy should be overcome if the artifact due to dye stacking is minimized. Therefore, an experiment was carried out in which the reference compartment contained 5 M urea and the sample compartment contained 0.3 M KCl. The concentration of dye in both cuvettes was 39.9 μM. Under these conditions, the dye in the reference cuvette will be almost completely in an unstacked state, and the dye in the sample compartment will be largely in the stacked state (see Figure 11). In the absence of GS, the recorded difference spectrum (solid line, Figure 20) is thus largely due to differences between stacked and unstacked dye molecules. If GS is now added to the sample cuvette (high-salt medium), the change in amplitude of the difference spectrum will reflect conversion of stacked free dye molecules to unstacked dye bound to GS. As shown in Figure 20, as the concentration of GS in the sample cuvette was increased, the amplitude of the difference spectrum decreased to a limiting value which reflects the difference between dye bound to GS (in 0.3 M KCl) compared to fully unstacked dye (in 6 M urea).

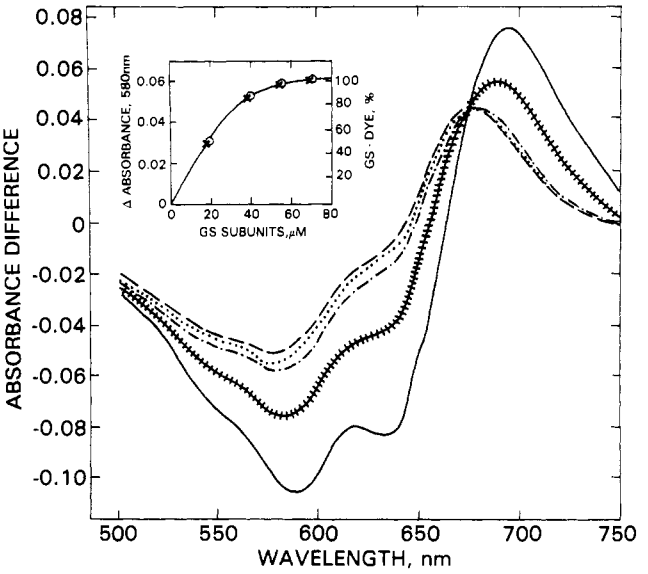

FIGURE 20: Effect of GS concentration on the difference spectrum between dye + GS in 0.3 M KCl vs. dye in 6 M urea. Both cuvettes contained 50 mM Hepes buffer (pH 7.4), 3.0 mM $MnCl_2$, and 39.9 μM dye. The reference cuvette contained 6 M urea, and the sample cuvette contained 0.3 M KCl. In addition, the sample cuvette contained either no GS (—) or 20.5 (∓), 39 (-·-), 56 (···), or 71 μM GS (- -). The temperature was 30 °C.

The presence of an isosbestic point at 642 nm attests to a monophasic binding mechanism. With the assumption that the change in absorbance at 680 nm reflects the amount of GS–dye complex formed, and the further assumption that the difference in absorbance at 680 nm in the absence and presence of 80 μM GS reflects 100% conversion of the dye to the GS–dye complex, the percent of dye in the complex at each GS concentration can be calculated. The relationship is shown in the inset of Figure 20 (open circles). These data are in excellent agreement with the percentages of dye that are calculated to be present in the GS–dye complex [see insert in Figure 20, (×)], using a K_d value of 0.55 μM (cf. Table I). Complications arising from dye stacking could be eliminated if the difference spectra are measured in 4 M urea, where the dye is almost completely unstacked (see Figure 11). Under these conditions, however, the subunits of GS are also completely dissociated. Therefore, in 4 M urea, the difference spectrum between dye and dye + GS reflects the binding of dye to dissociated GS subunits.

Figure 17B shows how the difference spectrum in 4 M urea, pH 7.5, varied when 20 μM GS-dissociated subunits was titrated with increasing amounts of dye. To avoid complications caused by the time-dependent nature of urea-promoted GS dissociation, the reaction mixtures also contained 3.3 mM EDTA to ensure rapid and complete subunit dissociation. In contrast to results obtained when GS was titrated with dye in the absence of urea (Figure 17A), the amplitude of the difference spectrum increased in a nearly linear manner as the concentration of dye was increased over the range of 20–90 μM (see insert, Figure 17B). Results of direct binding measurements under these same conditions (see Figure 18) established that the progressive increase in amplitude of the difference spectrum reflects the fact that at least 3.0 molecules of dye can be bound to each molecule of dissociated subunits of GS. That the binding of dye to the dissociated subunits is uncomplicated by dye stacking or by other factors is indicated by the fact that the difference spectra in Figure 17B exhibit a clean isosbestic point at about 641 nm. Other data in Figure 18 (open circles) show that dissociated subunits

produced by exposure of GS to EDTA and pH 8.7 can also bind more than two (probably at least three) molecules of dye per subunit. Multiple binding of dye to the GS subunits is therefore not dependent upon the presence of urea.

Discussion

The demonstration that uniquely different spectral perturbations are elicited by the binding of Cibacron Blue to taut, relaxed, oxidized, and dissociated forms of GS shows that, by means of difference spectroscopy, the dye can be used to monitor subtle conformational changes in protein structure. Moreover, by following the time-dependent changes in the difference spectrum, it is possible to study the kinetics of the conversion of one conformational state to another.

Recent studies by Maurizi & Ginsburg (1982) have demonstrated that GS is likely composed of six identical dimers arranged in a hexagonal bilayer and that dissociation of the enzyme probably proceeds randomly with the intermediate formation of decamers, octomers, hexamers, tetramers, and dimers, followed ultimately by conversion of the dimers to monomers. Once they are formed, the monomers and/or dimers may undergo reassociation to produce nondescript aggregates (Valentine et al., 1968; Woolfolk & Stadtman, 1967a,b). This and the earlier studies showing that urea causes complete dissociation of the enzyme to monomers (Woolfolk & Stadtman, 1967a,b; Shapiro & Stadtman, 1967) offer a reasonable explanation for the multiphasic kinetics observed in the dye vs. dye + GS difference spectrum in the presence of urea.

Because the difference spectrum between dye and some dye–enzyme complexes is very similar to that obtained between aqueous solutions of dye and dye dissolved in more nonpolar solvents, Thompson & Stellwagen (1976) suggested that the dye binds to hydrophobic regions on proteins. In the meantime, it was found (Edwards & Woody, 1979; Skotland, 1981; Subramaniam & Kaufman, 1980) that the difference spectrum obtained with some enzymes is very similar to that for dye vs. dye + 0.1 M KCl. In these cases, it was suggested that the difference spectrum reflects electrostatic interactions between dye and enzymes. Results of the present studies are consistent with both mechanisms and suggest further that in addition to hydrophobic and electrostatic interactions, the difference spectra are due in part to shifts in the equilibrium between dye monomers and dye aggregates. The difference spectrum obtained with either the relaxed or the taut form of GS is very similar to that obtained between dye in H_2O and dye in 0.2% Lubrol WS, supporting the view that hydrophobic sites on the enzyme may be involved in dye binding (cf. Figures 2 and 12). However, the difference spectrum obtained between dye and dye + taut GS is very similar to that obtained between dye and dye in 0.1 M KCl (cf. Figures 14 and 17A), suggesting that electrostatic interactions are likely involved. The likelihood that the difference spectrum is due in part to a large shift in the dye stacking equilibrium provoked by binding of the dye monomer to protein is supported by the fact that with saturating concentrations of dye only one molecule of dye is bound to each subunit of the taut form of GS (Figure 18); yet, the amplitude of the difference spectrum obtained when taut GS is titrated with dye increases well beyond the dye concentration required to saturate the enzyme (Figure 17A). As shown in Figure 19, this would be expected if only the dye monomer is capable of binding to the enzyme.

Finally, the possibility that dye–protein interactions may involve specific interactions between the dye and tryptophan residues in proteins must also be considered in view of the fact that tryptophan reacts directly with the dye, producing a substantial spectral perturbation (Figure 15). The fact that Cibacron Blue exists partly in an aggregated (stacked) state at concentrations greater than 5 μM is evident from the spectral difference observed between concentrated and dilute dye solutions in cuvettes of approximately short and long light paths, respectively (Figure 9). Edwards & Woody (1979) arrived at a similar conclusion based on the fact that a hypochromic dviation from Beer's law is observed in concentrations of Cibacron Blue above 5 μM.

The fact that 0.1 M KCl increases the rate of change in the difference spectrum between dye and dye + GS, in the presence of 3–4 M urea, is explained by the finding that inactivation (dissociation) of GS under this condition is retarded if the concentration of Mn^{2+} is increased. This suggests that in the presence of urea, high ionic strength decreases the affinity of the enzyme for Mn^{2+} and thereby facilitates conversion of the enzyme to the relaxed configuration, which is known to undergo rapid dissociation in the presence of urea (Woolfolk & Stadtman, 1967a,b; Ciardi et al., 1973).

Thompson & Stellwagen (1976) calculated the dissociation constants of dye–enzyme complexes from the relationship between the amplitude of the difference spectrum and the ratio of dye to enzyme. These dissociation constants and inhibitor constants based on the ability of nucleotides to quench the dye vs. dye + enzyme difference spectra were in agreement with K_i values determined by classical kinetic studies or by equilibrium binding measurements. The good agreement between these various methods is surprising in view of the fact that the difference spectra are due in part to the dye stacking phenomena. As shown here, when a given concentration of enzyme is titrated with dye, a change in the amplitude of the difference spectrum can occur well beyond the concentration of dye needed to saturate high-affinity binding sites on the enzyme. In the present study, the dissociation constant of the dye–GS complex ($K_d \approx 0.55$ μM) calculated from direct binding data is similar to that deduced from measurements of the ability of dye to inhibit the γ-glutamyltransferase activity of GS ($K_i = 0.85$ μM). There was also good agreement between values for the dissociation constants of the GS complexes with AMP, P_i, tryptophan, and AMP as calculated from dye binding competition experiments and the values derived from equilibrium binding data (Table II).

Subramanian & Kaufman (1980) showed that the difference spectrum between free dye and dye bound to dihydrofolate reductase from *Lactobacillus casei* is similar in some respects to the difference spectrum between dye dissolved in 0.1 M KCl or buffer and dye dissolved in a 1:1 mixture of dioxane and water. A very different difference spectrum is obtained with dihydrofolate reductase from chicken liver; this spectrum bears some resemblance to that obtained between dye in buffer vs. dye in 1.5 M NaCl. They deduce that the binding environment is predominantly "ionic" in the chicken liver enzyme and predominantly hydrophobic in the bacterial enzyme. In view of the fact that dioxane facilitates destacking of Cibacron Blue (Figure 11C), and the likelihood that high salt concentrations favor dye stacking (Figure 11D), one must consider also the contribution of dye stacking to the observed behavior.

Registry No. GS, 9023-70-5; ADP, 58-64-0; 5'-AMP, 61-19-8; tryptophan, 73-22-3; Cibacron Blue F_3GA, 12236-82-7.

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