

Binding Site Conformation Dictates the Color of the Dye Stains-all

A STUDY OF THE BINDING OF THIS DYE TO THE EYE LENS PROTEINS CRYSTALLINS*

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Yogendra Sharma, Ch. Mohan Rao, S. Chenchal Rao, A. Gopala Krishna,
Thayumanasamy Somasundaram, and Dorairajan Balasubramanian‡

From the Centre for Cellular and Molecular Biology, Uppal Road, Hyderabad 500 007, India

The interaction of the cationic carbocyanine dye Stains-all (1-ethyl-2-[3-(1-ethyl-naphtho[1,2-d]thiazolin-2-ylidene)-2-methylpropenyl]naphtho[1,2-d]thiazolium bromide) with the eye lens proteins crystallins has been studied. α - and γ -crystallins do not bind the dye, while β - and δ -crystallins do, consistent with the fact that the latter two proteins bind the calcium ion. β -Crystallin resembles parvalbumin in that it induces only the J-band of the bound dye. δ -crystallin, on the other hand, induces only the γ -band. Analysis of the metachromasia induced in the dye by these and other proteins suggests that Stains-all is responsive to the conformational status of the region to which it binds in a protein. The J-band of the dye is activated when it binds to a globular domain, and the γ -band is activated when it binds to a helical stretch of the protein.

Abnormal levels of calcium ion in the eye lens appear to induce opacity of the lens, and a variety of causative factors have been proposed (1-3), including Ca^{2+} -mediated aggregation and precipitation of the eye lens proteins, the crystallins (4, 5). We have recently compared (6) the ability of various crystallin molecules to bind Ca^{2+} and have found that while α - and γ -crystallins do not bind this ion, β - and δ -crystallins do, with affinity constants of 2600 and 4300 M^{-1} , respectively. Furthermore, we showed that δ -crystallin possesses the "EF-hand" or the "helix-loop-helix" calcium ion binding conformational motif in the region comprising residues 300-350 in its sequence. β -Crystallin, on the other hand, belongs to a single superfamily that also includes the calcium-binding protein S of the bacterium *Myxococcus xanthus* (7), and is particularly rich in anionic and polar amino acids that can bind Ca^{2+} , in the middle region of its sequence. We also noted that calcium binding alters the conformations of β - and δ -crystallins subtly and alters their photo-vulnerability differentially.

A convenient way of distinguishing between Ca^{2+} -binding proteins and others that has become available recently is the use of the metachromatic cationic carbocyanine dye Stains-all¹ (8-10). This dye stains several calcium-binding proteins blue or purple, and other proteins red or pink, thus suggesting that this dye might be able to identify potential Ca^{2+} -binding

proteins. For example, Urban *et al.* (11) have been able to identify a minor protein in the retinal rod outer segment as a calcium binding protein, since it stains blue with this dye. The problems associated with the self-aggregation of the dye have been minimized by the use of aqueous ethylene glycol as solvent, and it has been seen to display a high degree of sensitivity to variations in microenvironment, thus making it a probe of the microstructural features of proteins (12). Caday and Steiner (13) have also studied the interaction of Stains-all with free calmodulin and with the calmodulin-melittin complex, and concluded that the complex formation involves the α -helical connecting bridge joining the N- and C-terminal lobes of the calmodulin. It has also been found (14) that different calcium binding proteins interact with Stains-all differently, and yield different spectral bands. At high dye:protein molar ratios, calmodulin, troponin C, and parvalbumin complex with the dye similarly and yield what is termed (15-17) the J-band in the 600-650 nm region. As the dye:protein ratio is decreased, the J-band is lost in the former two proteins, yielding the β - and the γ -bands at 535 and 500-510 nm, respectively; with parvalbumin, the J-band is retained at all stoichiometries. Also, while the J-band is replaced by the β - and γ -bands upon the addition of Ca^{2+} to the dye complexes of calmodulin and troponin C, with parvalbumin, the J-band is simply lost and the bound dye is released. This dye thus appears to enable one to distinguish between Ca^{2+} -binding proteins from others, and also to probe possible structural differences among Ca^{2+} -binding proteins themselves. We have explored these possibilities in the case of the crystallins in this paper. We find that with β -crystallin, it is the J-band that is activated upon Stains-all binding, while with δ -crystallin it is the γ -band that gets activated. The addition of calcium ions to these complexes abolishes these spectral bands. In contrast, α - and γ -crystallins are not able to induce any spectral changes in the dye. Based on these and other results, it appears possible to propose "conformational selection rules" for color induction in the dye Stains-all, when it is bound to proteins and other biopolymers.

EXPERIMENTAL PROCEDURES

Materials— α , β , and γ -crystallins were purified from rat lenses by gel filtration methods described earlier (18). In a variation of the old method (19), δ -crystallin was purified from 3-day-old chick lenses by Sephacryl S-200 chromatography in Tris buffer, pH 9.1, followed by elution in a thiol-Sepharose affinity column, as described earlier (6). The dye Stains-all was obtained from Aldrich. All other chemicals were of the highest purity available.

Spectrometry of the Protein-Dye Complexes—The protocol used for preparing the dye solutions and the protein-dye complexes was the same as described by Caday and Steiner (12). The Stains-all solutions were prepared by dissolving the dye in ethylene glycol to about 0.5 mM and the actual concentration of the dye determined based on a molar extinction coefficient value at 578 nm of 1.13×10^5 in ethylene glycol. In order to make the complexes, crystallins were dissolved in

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‡ To whom correspondence should be addressed.

¹ The abbreviations used are: Stains-all, 1-ethyl-2-[3-(1-ethyl-naphtho[1,2-d]thiazolin-2-ylidene)-2-methylpropenyl]naphtho[1,2-d]thiazolium bromide; MOPS, 3-(*N*-morpholino)propanesulfonic acid.

2 mM MOPS buffer, pH 7.2, containing 30% ethylene glycol, to which an aliquot of the stock solution of the dye was added, and the mixture incubated typically for an hour in the dark. The use of 30% ethylene glycol helps in preventing the time-dependent self-aggregation of Stains-all in aqueous solution, a complication that would have interfered in the interpretation of spectral changes. Also, any complications that might arise due to photobleaching of the dye were avoided by working, as far as possible, in the dark or in very low levels of light (12). Absorption spectra were recorded using a Hitachi model 150-20 spectrophotometer, while circular dichroism (CD) spectra were recorded using a Jasco model J-20 spectropolarimeter. The concentrations of the crystallins were obtained spectrophotometrically, using the published extinction coefficient value of each of them. Molecular ellipticities are expressed in terms of the dye molarity, using the extinction coefficient of the dye mentioned above.

RESULTS AND DISCUSSION

Several different types of complexes of Stains-all with proteins and other polyelectrolytes have been distinguished, based on the position of their absorption spectral maxima (15-17). The complexes, together with their absorption maxima, are the α (575 nm), β (535 nm), γ (500-510 nm), $\beta\alpha$ (550 nm), S (470 nm), and J (600-650 nm) species. The free dye in the monomeric form absorbs at 575 nm, or is in the α state, in organic solvents or in very low concentrations in water. In 30% ethylene glycol in water, the free dye itself absorbs at 535 and 575 nm, *i.e.* displays the β -band and the α -band, respectively. Fig. 1 shows that α -crystallin and γ -crystallin do not significantly affect the spectral features of Stains-all, regardless of the dye:protein molar ratio chosen. As the dye:protein ratio is lowered, α -crystallin appears to broaden the β -band and generate the band around 500-510 nm as a minor component. γ -Crystallin does not appear to interact with the dye at all. Thus, these two proteins do not bind Ca^{2+} , nor do they seem to have anionic sites properly disposed to bind Stains-all.

The situation with β -crystallin is quite different. As Fig. 2 reveals, the dye displays substantial spectral shifts in the presence of this protein. The J-band is seen to grow at the

cost of the α - and the β -bands, as the dye:protein ratio is progressively decreased. The J-band is broad, with the maximum around 640 nm, which seems to attain maximum intensity at a dye: β -crystallin ratio of about 4. Upon further decreasing this ratio, the J-band loses its intensity and shifts its maximum a little to the red. That the dye is bound proximally to the protein and interacts electronically with it is clear from *panel B* of Fig. 2, which shows that optical activity is induced in the achiral dye molecule. A biphasic CD spectrum develops in the J-band region, which levels off in value at about a dye:protein ratio of 4. (Interestingly, the α -band and the β -band of the dye are still optically inactive, consistent with the interpretation that these arise from the free or unbound dye.) In all these features, β -crystallin behaves in a fashion similar to the calcium-binding protein parvalbumin, and somewhat different from calmodulin and troponin C (14). Like parvalbumin, β -crystallin does not generate the γ -band of the dye at low dye:protein ratios, in place of the J-band. However, the maximum spectral effects on the bound dye are seen with β -crystallin at a ratio of 4, while no leveling off seems to occur with parvalbumin.

Addition of calcium ions to a solution of the β -crystallin-Stains-all complex is illustrated in Fig. 3. While 4 mM Ca^{2+} affects the spectrum only marginally, 40 mM Ca^{2+} abolishes the J-band and restores the intensities of the α - and the β -bands to the values seen with the free dye case. *Panel B* of Fig. 3 shows that CD spectroscopy is even more sensitive to this effect of calcium ions; addition of 4 μM Ca^{2+} reduces the induced optical activity of the dye to half its value, while 3 mM CaCl_2 is sufficient to abolish the CD bands of the β -crystallin-Stains-all complex in this region of the spectrum. In this respect again, β -crystallin behaves similar to parvalbumin.

The behavior of δ -crystallin toward Stains-all is quite different from that of β -crystallin. As Fig. 4 shows, this protein does not induce the J-band of the dye at all, but instead it shifts the β -band to the blue and converts it into the γ -band

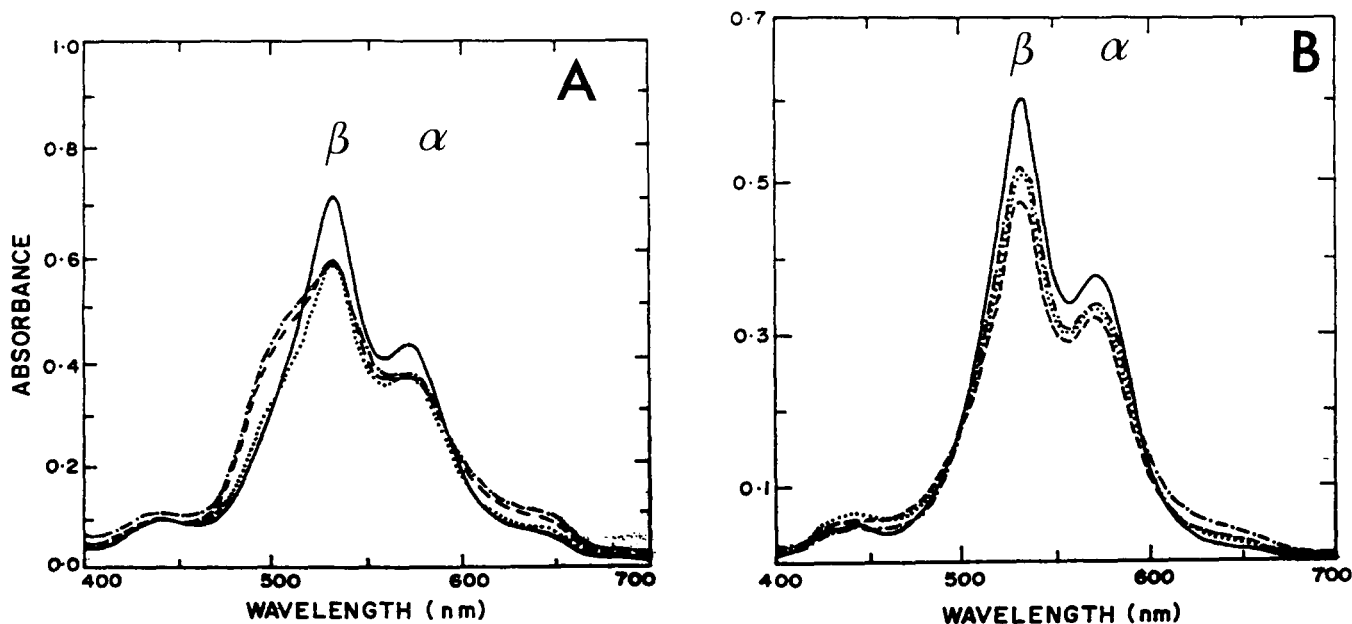


FIG. 1. *A*, the absorption spectrum of a 10.5 μM solution of the dye Stains-all in 2 mM MOPS, 30% ethylene glycol, pH 7.2, in the absence and presence of rat lens α -crystallin: dye alone (—); dye:protein mole ratio 150 (....); 50 (----); and 25 (-.-.-). This same buffer solution was used in all spectral studies illustrated in all the other figures as well. *B*, the absorption spectrum of a 9.37 μM solution of Stains-all in the absence and presence of rat lens γ -crystallin: dye alone (—); dye:protein mole ratio 2.4 (-.-.-); 1.6 (----); and 0.8 (....).

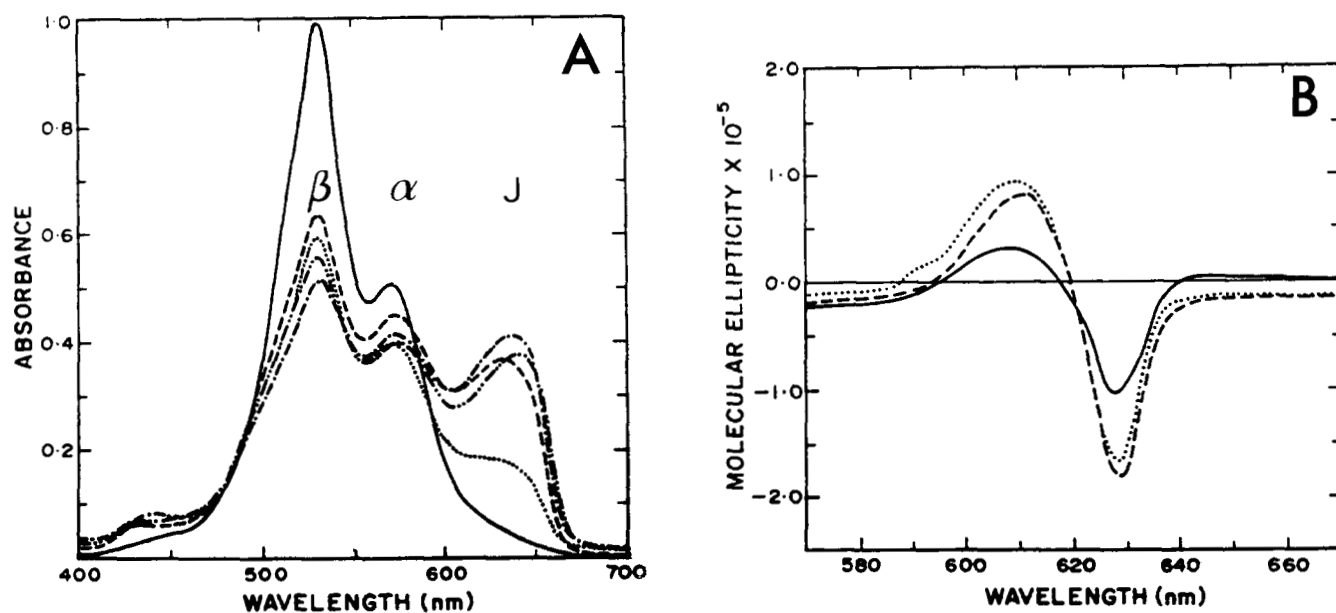


FIG. 2. A, the effect of rat lens β -crystallin on the absorption spectrum of 15 μ M Stains-all solutions: dye alone (—); dye:protein molar ratio 11.5 (....); 5.75 (---); 3.72 (-.-.-); and 2.8 (-.-.-.-). B, CD spectra of 15 μ M Stains-all in the presence of rat lens β -crystallin: dye:protein mole ratio 5.75 (—); 3.72 (....); and 2.8 (---).

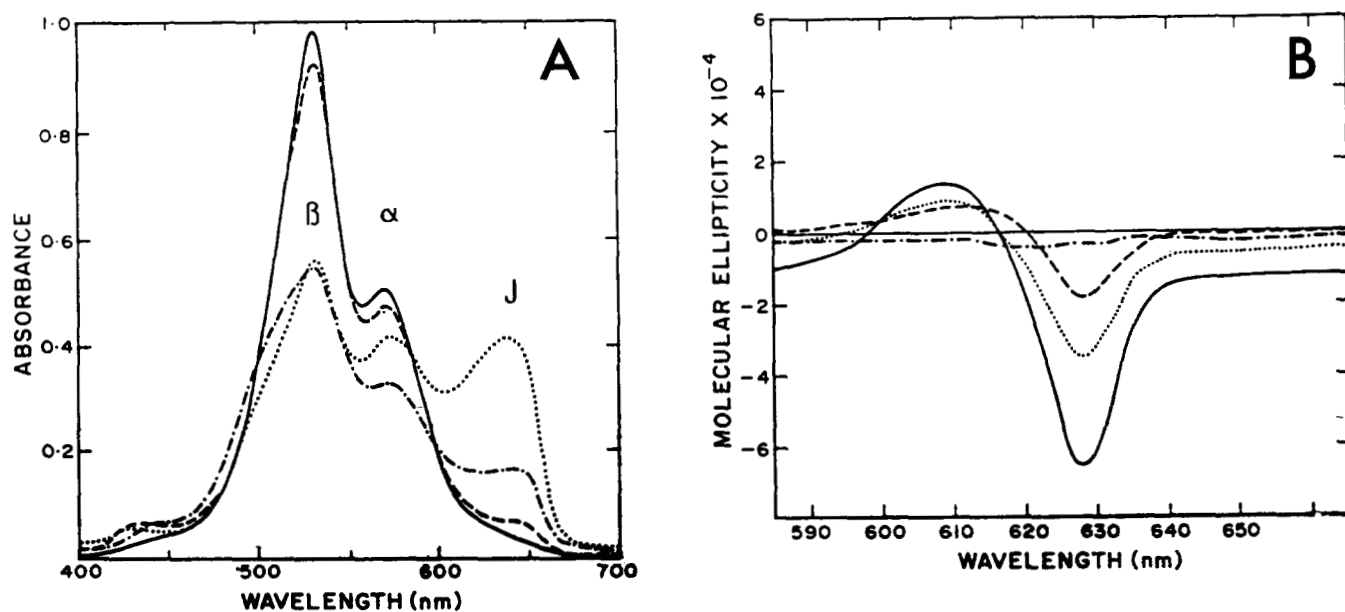


FIG. 3. A, effect of the addition of calcium ions on the absorption spectrum of the Stains-all- β -crystallin complex. The concentration of the dye was 15 μ M, and the dye:protein mole ratio was 3.72. Curve is the spectrum of the complex alone, with no added calcium; curve --- is for the complex in the presence of 4 mM CaCl_2 ; and curve -.-.- in the presence of 40 mM CaCl_2 . The spectrum of the dye alone in the buffer is shown by curve —. B, effect of the addition of calcium ions on the CD spectrum of the Stains-all- β -crystallin complex. The dye concentration was 15 μ M, and the dye:protein ratio was 3.72. Curve — is the CD spectrum of the complex alone, with no added calcium; curve is for the complex in the presence of 4 μ M CaCl_2 ; curve --- is in the presence of 15 μ M CaCl_2 ; and curve -.-.- is in the presence of 3 mM CaCl_2 .

with a band maximum value of 505 nm. The α -band of the dye near 570 nm is not shifted at all. In this behavior, δ -crystallin differs from the usual calcium-binding proteins and resembles bovine albumin, hemoglobin, chymotrypsinogen, and polyglutamic acid below pH 4, the so called γ -group of macromolecules (15). As can be seen from Fig. 4B calcium ions restore the spectrum of the free dye, suggesting that in the presence of calcium ions, δ -crystallin binds the dye very weakly or not at all. Similar reversal is noted in the CD spectral profiles of the dye-protein complex, as Fig. 5 reveals. Binding to δ -crystallin activates the optical activity of the γ -

band of the dye in a biphasic manner in the 470–540 nm region, and 0.2 mM CaCl_2 is able to reduce the intensities of these CD bands substantially. This behavior of δ -crystallin is particularly unexpected not only because it binds to the calcium ion better than β -crystallin, but also because it possesses the EF-hand or the helix-loop-helix conformational motif in the region comprising residues 300–350 of its sequence (6). Yet its staining behavior resembles that of several proteins that are not noted for their Ca^{2+} -binding ability.

We thus note that there is not one but three different types of behavior exhibited by Ca^{2+} -binding proteins toward Stains-

FIG. 4. A, the absorption spectrum of a 8.9 μM solution of the dye Stains-all in the absence and presence of chick lens δ -crystallin. Dye alone (—); dye:protein mole ratio 318 (....); 159 (---); and 40 (----). B, effect of the addition of calcium ions on the absorption spectrum of the Stains-all- δ -crystallin complex. Curve -- is obtained upon the addition of 47 μM CaCl_2 ; curve upon the addition of 0.2 mM CaCl_2 ; and curve — upon the addition of 1.8 mM CaCl_2 .

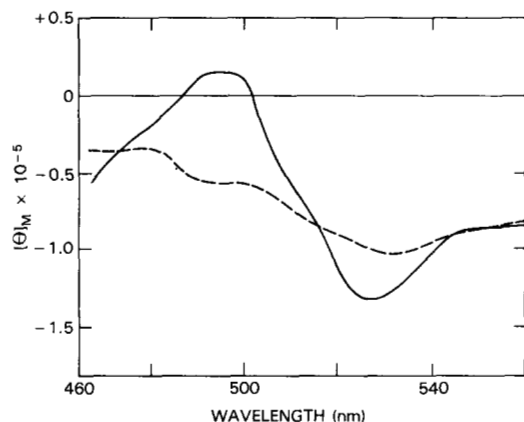
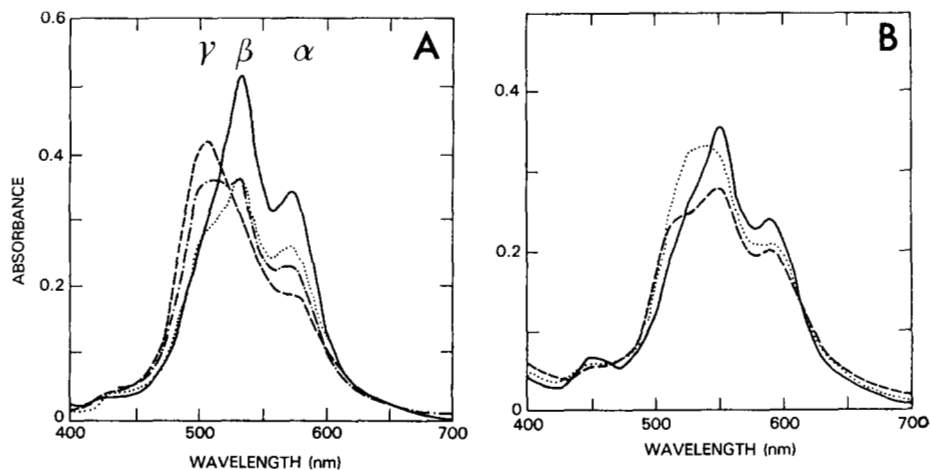


FIG. 5. Effect of the addition of CaCl_2 on the CD spectrum of the Stains-all-chick lens δ -crystallin. Curve — is that of the complex at a dye:protein mole ratio of 59, and curve ---- is obtained upon the addition of 0.2 mM CaCl_2 to the complex.

all. One type is represented by calmodulin and troponin C, which induce the J-band at high dye:protein ratios; as this ratio is lowered (or when Ca^{2+} is added), the J-band is replaced by the β - and γ -bands. The second type is displayed by parvalbumin and β -crystallin, where only the J-band is seen at all dye:protein ratios; addition of Ca^{2+} abolishes this band and restores the spectrum of the free dye. The third type of spectral perturbation is exhibited by δ -crystallin. Here, only the γ -band of the dye is activated, and Stains-all does not stain this calcium-binding protein blue. We believe that the clue to this diverse behavior of these proteins lies in their conformational status. Calmodulin (20) and troponin C (21, 22) are dumbbell-shaped structures with two globular lobes connected by a long, exposed helical stretch. The EF-hand Ca^{2+} -binding sites are in the globules, and the overall helical content of the molecules about 65%. Parvalbumin (23, 24) differs from these two proteins in that it consists of only a single globular unit that contains two Ca^{2+} -binding sites, and there is no long helical strand. Caday *et al.* (14) have earlier argued that it is this conformational difference that is responsible for the difference in the behavior of calmodulin and troponin C on the one hand, and parvalbumin on the other. They have proposed that the J-band is induced in Stains-all when it binds to the common structural element, namely the globular lobe, and that the γ -band arises when the dye is bound to the strand connecting the lobes, *i.e.* the long helical stretch.

Interestingly, such as assignment of the spectral bands of

the bound dye to the conformational status of the substrate raises the possibility of whether a simple water-soluble helical peptide chain that binds Stains-all would activate the γ -band. We note in this connection that polyglutamic acid below pH 4, under which conditions it is folded completely in the right handed α -helical conformation, indeed does so. On the other hand, above pH 4, when it turns into a randomly coiled form, it generates neither the γ - nor the J-band but the $\beta\alpha$ -band centered around 550 nm. Polyaspartic acid, which does not adopt the helical fold (25), generates the β -band (15). It would also be consistent with what we have observed in the case of Stains-all bound to chick lens δ -crystallin, since the conformation in solution of this molecule is over 80% right-handed α -helical form (26). While only high resolution NMR spectral analysis or crystal structure would provide more definitive information, it would appear appropriate in this context, as of now, to consider the chick lens δ -crystallin chains as a calcium ion binding polymer which is a long exposed stretch of α -helix which has one EF-hand motif that binds the metal ion, which therefore generates not the J-band but the γ -band in the dye. Such an analysis would also explain why the other Ca^{2+} -binding crystallin, namely β -crystallin, induces only the J-band and not the γ -band in the bound dye. β -Crystallin adopts a "greek key" motif bilobal globular structure, where the secondary structural order is not the α -helix but the antiparallel β -pleated sheet form (27, 28). For the present purpose, therefore, this molecule may be thought of as having the calcium-binding globule which would generate the J-band, but no helical stretch that generates the γ -band.

These observations prompt us to suggest that the absorption spectrum of the bound dye Stains-all is governed by what may be called "conformational selection rules," summarized below.

(i) The J-band occurs when the dye is bound to the anionic sites present in the globular or compact conformations of the protein. In proteins where the binding region occurs elsewhere in the structure, the J-band will not be activated and the protein will not stain blue.

(ii) The γ -band of the dye occurs when the dye is bound in a long and exposed helical region of the polymer. Ca^{2+} -binding proteins that do not contain binding sites in a helical segment may not induce this band.

(iii) It may not even be necessary that the protein or the macromolecule be a calcium binding substrate; it may suffice that it contain anionic charges which enable the binding of the cationic dye Stains-all. It may then generate the J-band or the γ -band, depending on (i) and (ii) above.

It would be of interest to test the validity of the selection

rules given above by studying the behavior of more proteins and other related biopolymers. Analysis of published reports lends credence to these proposals. For example, bovine brain S-100 proteins generate the J-band in Stains-all, which is replaced by the γ -band upon calcium addition (29); these proteins contain two largely homologous subunits α and β , and are folded about 60% in the α -helical conformation (30). These proteins thus resemble calmodulin in staining behavior. So does the activation segment of pig procarboxypeptidase A (31), which is also thought to have the calmodulin-type conformational pattern. The phosphoproteins, phosphovitin and caseins, which stain blue (32), would be considered to fall under rule (i) above. They are indeed non-helical proteins (33), and do not have well defined or special Ca^{2+} -binding motifs. Sialoglycoproteins also generate the J-band in this dye (34), and their conformational analysis would be of interest, as also their calcium binding propensities. It might very well turn out that the dye Stains-all discriminates not calcium-binding proteins from others, but on a more basic level, helical or rod-like binding sites from globular binding sites in the substrate biopolymer.

(iv) A substrate that does not have anionic sites properly disposed so as to enable it to bind Stains-all will not induce any color changes in the dye. α - and γ -crystallin represent this trivial case.

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