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Investigation into the binding of dyes within protein crystals

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PDB references: thaumatin with thioflavin, 6c6w; thaumatin with xylene cyanol, 6e0d

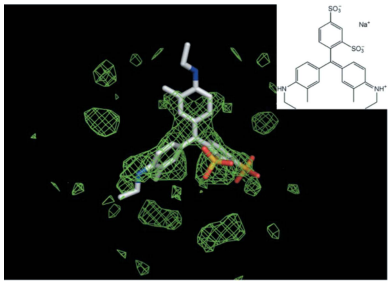
Supporting information: this article has supporting information at journals.iucr.org/f

It was found that the crystals of at least a dozen different proteins could be thoroughly stained to an intense color with a panel of dyes. Many, if not most, of the stained protein crystals retained the dyes almost indefinitely when placed in large volumes of dye-free mother liquor. Dialysis experiments showed that most of the dyes that were retained in crystals also bound to the protein when free in solution; less frequently, some dyes bound only in the crystal. The experiments indicated a strong association of the dyes with the proteins. Four protein crystals were investigated by X-ray diffraction to ascertain the mode of binding. These were crystals of lysozyme, thaumatin, trypsin inhibited with benzamidine and satellite tobacco mosaic virus. In 30 X-ray analyses of protein crystal–dye complexes, in only three difference Fourier maps was any difference electron density present that was consistent with the binding of dye molecules, and even in these three cases (thaumatin plus thioflavin T, xylene cyanol and *m*-cresol purple) the amount of dye observed was inadequate to explain the intense color of the crystals. It was concluded that the dye molecules, which are clearly inside the crystals, are disordered but are paradoxically tightly bound to the protein. It is speculated that the dyes, which exhibit large hydrophobic cores and peripheral charged groups, may interact with the crystalline proteins in the manner of conventional detergents.

1. Introduction

An interesting observation emerged from a class crystallization experiment carried out during the Cold Spring Harbor Laboratory Course in X-ray Methods in Structural Biology (Cold Spring Harbor, New York, USA). The protein lysozyme was co-crystallized by vapor diffusion (McPherson & Gavira, 2014; McPherson, 1982, 1999) with a series of common dye molecules. This resulted in a vast number of richly colored crystals, indicating that the dye molecules had been incorporated. The same final result was obtained, with some exceptions, when the dyes were simply added directly to the mother liquors of existing lysozyme crystals and allowed to diffuse into the crystals over several days. More intriguing was the observation that if the dyed crystals were gathered together in a large volume (several millilitres) free of any of the dyes, after 10 d most (but not all) of the crystals appeared to retain their dye color. This suggested that the dye molecules were either bound directly to the lysozyme molecules in the crystal, that they were trapped in lattice interstices formed by intramolecular contacts defining the crystal, or both.

The intention of the experiments described here was to explore these observations further and determine (i) how long different protein crystals would retain various dyes before the dyes diffused away and the crystals became clear, (ii) whether



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the effect was confined to lysozyme crystals or whether it was to be expected of other protein crystals and (iii) the nature of the binding of the dye molecules in the protein crystals. This last question was addressed using X-ray crystallography.

Most of the dyes used in the Cold Spring Harbor course were arbitrary histological stains, about a dozen in number, gleaned from neighboring laboratories. This set was expanded to about 40 dyes in the experiments described here and included other types of stains, including some that are used to dye textiles and other biologically derived materials.

A representative selection of dyes is shown in Fig. 1. A common feature of the dyes is an extensive, conjugated system of aromatic components that serves as the chromophore and produces the distinctive color of the dye. The chromophore is highly hydrophobic and would naturally be insoluble in aqueous solution. The dyes are made water-soluble (although some are barely so) by the attachment of charged hydrophilic groups. These are usually amino groups, quaternary amines or sulfonate groups. Dyes fall roughly into two categories: those carrying positive charges (at neutral pH), termed basic dyes, and those carrying negative charges, termed acidic dyes (Horobin & Kiernan, 2002). For some dyes the ionizable groups can exist in protonated or nonprotonated states, and the specific state affects the electronic structure of the chromophore, thereby causing it to change from one color to another. These are known as pH-sensitive dyes and have been widely used in chemistry and biochemistry as pH indicators. For example, *m*-cresol purple is yellow below about pH 7 but is blue above.

Other dyes are sensitive to reduction or oxidation and change color, or lose color, in the presence of a reductant such as bisulfite or an oxidant such as hydrogen peroxide. These are termed redox-sensitive dyes and are also used as indicators. The dyes investigated in our experiments included all of these types. It might also be noted that a number of the dyes, for example bromophenol blue, carry several Br atoms that should be readily visible in electron-density maps. Another, erythrosine B, a derivative of fluorescein, carries four I atoms, which should be even more evident.

The modes of binding of dyes to biological macromolecules, including proteins, nucleic acids, polysaccharides and lipids, is not clear at the molecular level and examples of protein–dye complexes in the literature are rare. Most examples that are found are not general in nature but involve the substitution of a coenzyme by a dye (for examples, see McArdell *et al.*, 1987; Wassarman & Lentz, 1971) or the intercalation of a dye, such as acriflavin or acridine, among the stacked bases of RNA or DNA (Kennard & Hunter, 1989).

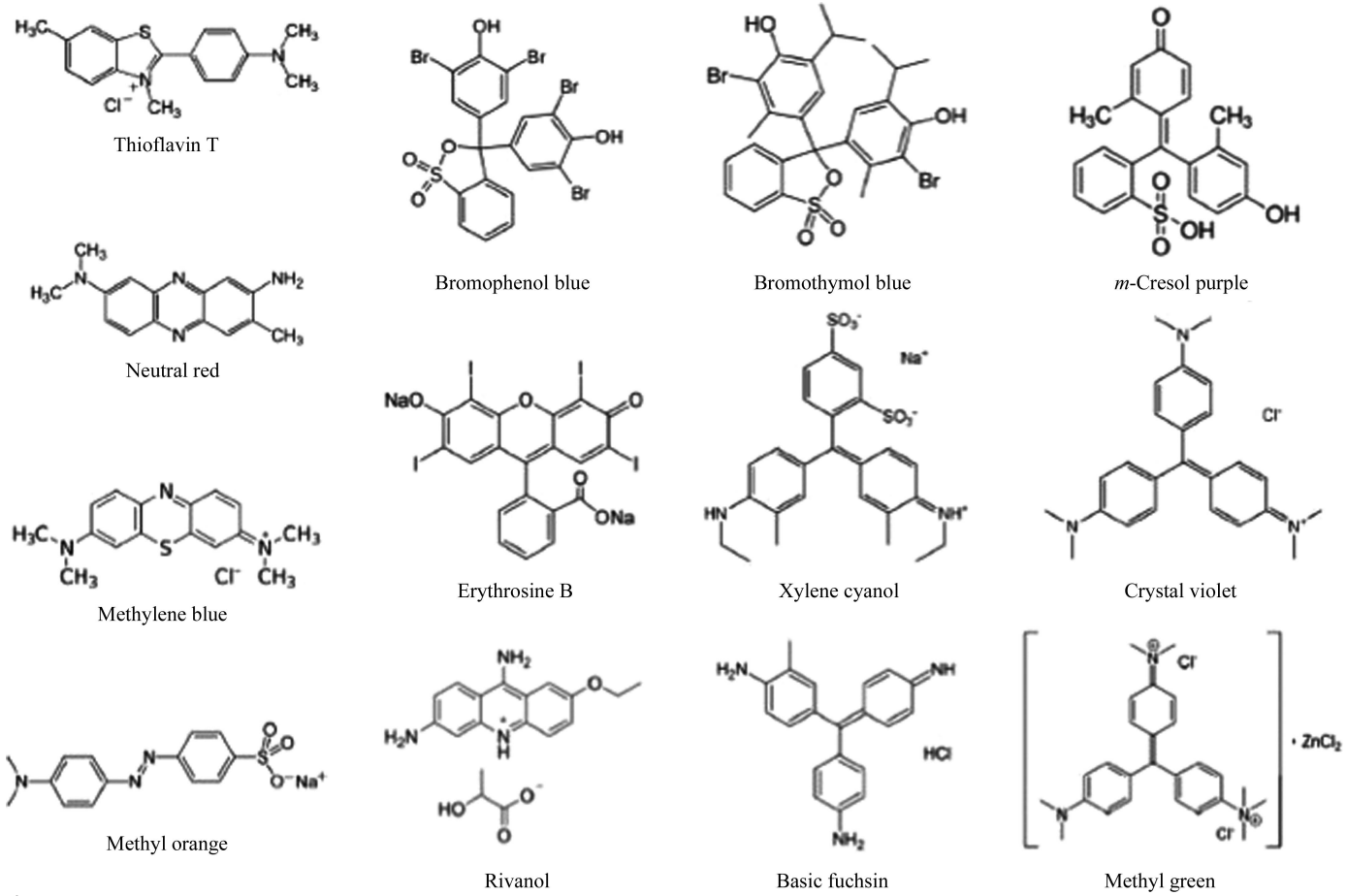


Figure 1
A selection of chemical structures from the set of 28 dyes included in this investigation. All are characterized by an aromatic, hydrophobic core with associated ionizable groups.

Table 1
Dyes and stains investigated.

Dye	Molecular weight	pH or redox indicator
Bromophenol blue (four Br atoms)	670	pH
Rivanol (ethacridine lactate)	343	
Erythrosine B (four I atoms)	880	
Xylene cyanol	539	
Nitro blue tetrazolium chloride	818	
Evans blue (very low solubility)	960	
Chicago Sky Blue 6B (very low solubility)	993	
Brilliant Blue R (very low solubility)	826	
Basic fuschsin	338	Redox
Congo red (chlorophenol red)	697	pH
Neutral red	289	pH
Thioflavin T	319	
Methyl orange	327	pH
Coomassie Brilliant Blue	826	
m–Cresol purple	382	pH
Crystal violet	408	Redox
Methylene blue	320	Redox
Safranin orange	351	
Azure blue	306	
Methyl green	653	Redox
Bromothymol blue (two Br atoms)	624	Redox
Bromocresol purple (two Br atoms)	540	pH
Phenol red	354	pH
Bromocresol green (four Br atoms)	698	pH
Dimethyl yellow (low solubility)	225	pH
Acriflavin	260	
Trypan blue	873	
Solvent Blue 38 (Luxol, contains Cu^{2+})	696	Redox

The current understanding of dye binding or staining of biological materials, tissues and microorganisms is that binding occurs chiefly through electrostatic interactions produced by the positively charged groups (basic dyes) or negatively charged groups (acidic dyes) on the molecules (Horobin & Kiernan, 2002; Gurr, 1971). Thus, basic dyes such as eosin are commonly used by histologists to stain nucleic acids, for example chromosomes, while acidic dyes may stain other organelles such as lysosomes.

2. Experimental

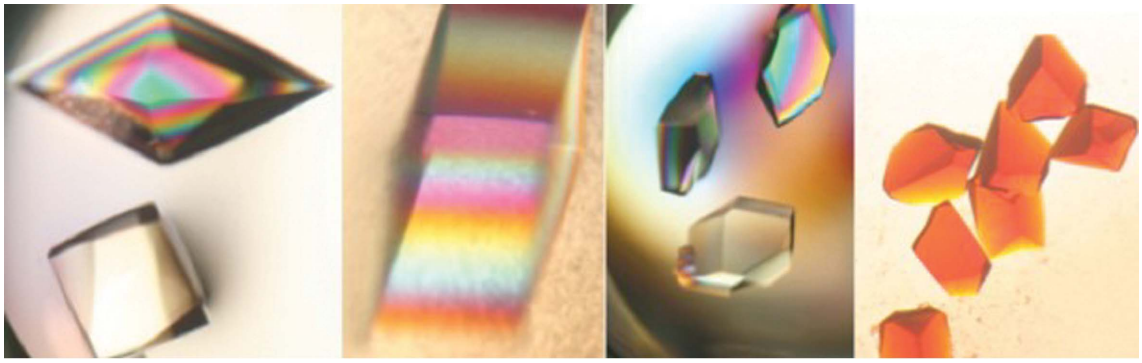
The dyes used in our experiments are listed in Table 1 along with their molecular weights and whether the dyes are pH or redox indicators. If not, whether they contain heavy atoms is noted. Four macromolecular crystals were investigated using X-ray crystallography as potential complexes with dyes: these (shown in Fig. 2) were tetragonal lysozyme, tetragonal thaumatin, orthorhombic crystals of bovine trypsin inhibited with benzamidine, and crystals of satellite tobacco mosaic virus (STMV). The crystals for X-ray analysis were generally about 0.5 mm in most dimensions. All crystals were produced by sitting-drop vapor diffusion in 24-well Cryschem plates (Hampton Research, Aliso Viejo, California, USA) with mother-liquor droplets of 8–16 µl and reservoirs of 0.6 ml. Crystallization conditions for each of the protein crystals and the unit cells are as follows.

2.1. Thaumatin crystals

The sweet protein thaumatin (Sigma–Aldrich) was dissolved to a concentration of 50 mg ml^{-1} in 0.1 *M* HEPES pH 6.5. Drops of a 1:1 mixture of the protein solution and the reservoir solution were equilibrated using sitting-drop vapor diffusion with 0.6 ml reservoir solution. The reservoir solution was varied between 0.5 and 1.5 *M* unbuffered sodium potassium tartrate. The crystals belonged to space group $P4_12_12$, with unit-cell parameters $a = b = 58.6$, $c = 151.8$ Å, $V_M = 2.93$ Å^3 Da^{-1} and 58% solvent content.

2.2. Crystals of trypsin inhibited with benzamidine

Purified bovine trypsin (Sigma–Aldrich) was dissolved to a concentration of 40 mg ml^{-1} in 0.1 *M* Tris–HCl buffer containing 4 m*M* calcium chloride and 0.2 *M* benzamidine. Large crystals were grown over a week by vapor diffusion in sitting-drop Cryschem plates. The sample drops consisted of a 1:1 mixture of the protein solution with 1.35–1.75 *M* ammonium sulfate in water. They could also be grown by substituting sodium malonate in the range 35–43% saturation at pH 7 for the ammonium sulfate. The crystals belonged to space group $P2_12_12_1$, with unit-cell parameters $a = 54.7$, $b = 58.3$, $c = 67.6$ Å, $V_M = 2.37$ Å^3 Da^{-1} and 48% solvent content.



(*a*) (*b*) (*c*) (*d*)

Figure 2
Crystals of the four proteins examined in this investigation. They are (*a*) tetragonal thaumatin, (*b*) orthorhombic trypsin plus benzamidine, (*c*) tetragonal lysozyme and (*d*) satellite tobacco mosaic virus. The crystals in (*a*)–(*c*) are greater than 1 mm in their dimensions and those in (*d*) are about 0.25–0.4 mm in size. The cystals in (*a*)–(*c*) are viewed with polarized light; the crystals in (*d*) are stained with methyl orange.

2.3. Tetragonal lysozyme crystals

Hen egg-white lysozyme (Sigma–Aldrich) was dissolved to a concentration of 100 mg ml^{-1} in H_2O. Drops of the protein solution mixed in a 1:1 ratio with reservoir solution were equilibrated by sitting-drop vapor diffusion against reservoir solutions consisting of 6–8%(*w*/*v*) sodium chloride buffered with 0.1 *M* sodium acetate pH 4.8 or 0.1 *M* HEPES pH 6.5–7.5. The largest crystals grew at room temperature over the period of a week from the samples at higher pH.

The crystals belonged to space group $P4_32_12$, with unit-cell parameters $a = b = 78.7$, $c = 38.6$ Å, $V_M = 2.09$ Å^3 Da^{-1} and 41% solvent content.

2.4. Satellite tobacco mosaic virus crystals

STMV was prepared from TMV–STMV co-infected tobacco leaves as described elsewhere (Koszelak *et al.*, 1989). The virus was dissolved to a concentration of 4–8 mg ml^{-1} in 0.1 *M* phosphate buffer pH 6.0. Drops consisting of 1:1 mixtures of STMV solution with reservoir solution were equilibrated by vapor diffusion with reservoirs containing ammonium sulfate at 10–20% saturation. The crystals belonged to space group *I*222, with unit-cell parameters $a = 174.3$, $b = 191.8$, $c = 202.5$ Å, $V_M = 2.26$ Å^3 Da^{-1} and 46% solvent content.

We found that co-crystallization of dyes with the proteins was, in general, not necessary as exposure of existing crystals to dye for 48 h or longer was usually sufficient to saturate the crystals. Diffusion of the dye was therefore used to form the crystalline complexes for lysozyme, STMV and trypsin inhibited with benzamidine. The protein–dye complex crystals of thaumatin, however, were all made by co-crystallization. The presence of dye in the mother liquors did not significantly affect crystallization in general, although instances were observed where crystallization failed in the presence of some dyes. There were also a number of cases in which the dye could not be diffused into existing crystals, particularly large dyes with higher molecular weights such as Evans blue and trypan blue. Virtually no instances were seen where dye diffused into the crystals and produced visible damage as long as the dyes were dissolved in matching mother liquor lacking protein. The presence of dyes in the crystals did not appear to affect cryogenic flash-cooling.

Manipulation of dye solutions and mother liquors, exchange of mother liquors and most other handling of small volumes of liquid was carried out using gel-loading pipette tips and 10 µl pipettors. When it was necessary to completely remove a mother liquor before modification of conditions, the mother liquor was drawn away using a micropipette and this was followed by additional drying using absorbent paper wicks (Hampton Research). Manipulation and transfers of individual crystals, where necessary, were carried out using cryoloops and meshes of 50–300 µm in diameter (MiteGen, Ithaca, New York, USA). Observation and photography utilized an Olympus SZX12 zoom-lens microscope with an adaptor coupling it to a Canon EOS digital camera. Manipulations of mother liquors and crystals were usually performed with a lower magnification zoom-lens dissecting microscope.

Table 2
X-ray diffraction/difference Fourier analyses of complexes.

Lysozyme	STMV	Thaumatin	Trypsin
Bromophenol blue	Luxol Blue	Xylene cyanol	Luxol blue
Rivanol	Methylene blue	Neutral red	Toluidine blue
Erythrosine B		Thioflavin T	Methylene blue
Xylene cyanol		*m*-Cresol purple	Bromocresol purple
Nitro blue tetrazolium		Methylene blue	Thioflavin T
Basic fuchsin		Bromophenol blue	*m*-Cresol purple
Chlorophenol red		Erythrosine B	
Neutral red		Rivanol	
Methyl orange		Evans blue	
Crystal violet		Tetracycline	
Methylene blue		Nitro blue tetrazolium	
Flavin mononucleotide			

Microdialysis experiments were conducted on nine different proteins, including those for which X-ray analyses were carried out, to evaluate the retention of the dye molecules by proteins in solution. For these experiments, dialysis chambers and retaining rings were made from 1.5 ml Eppendorf microcentrifuge tube rings and caps. The rings snapped over the dialysis membrane-covered caps, the interior cavities of which held about 200 µl protein solution. The protein–dye mixtures were dialyzed against three to four changes of 1 l deionized water for a period of not less than 60 h. They were evaluated for dye retention by visual inspection for color.

Most X-ray diffraction data collection was carried out at room temperature on crystals mounted in the conventional manner (McPherson, 1982) in 0.7–1.0 mm quartz capillaries containing a little mother liquor. Data at cryogenic temperatures were collected on beamline 8.3.1 at the Advanced Light Source. The dyes investigated by X-ray crystallography in complex with proteins are shown in Table 2. Room-temperature data collection was carried out using a Rigaku MicroMax-007 HF generator operated at 40 kV and 30 mA with a Rigaku Saturn 944+ CCD detector. X-ray data were processed using *d*TREK* (Pflugrath, 1999), *MOSFLM* (Battye *et al.*, 2011) and *XDS* (Kabsch, 2010). Data from native crystals containing no dye were also collected so that $F_o - F_o$, $F_o - F_c$ and $2F_o - F_c$ difference Fourier maps could be computed. Visualization of the maps and models employed *Coot* (Emsley & Cowtan, 2004) and images were prepared with *PyMOL* (DeLano, 2002). The data extended to beyond 2.4 Å resolution in all cases and to beyond 1.8 Å resolution in some. Structures were determined by molecular replacement using *Phaser* (McCoy *et al.*, 2007). The search models were PDB entry 1iee for lysozyme (Sauter *et al.*, 2001), PDB entry 1thw for thaumatin (Ko *et al.*, 1994), PDB entry 3t25 for trypsin (Marshall *et al.*, 2012), and PDB entries 4nia, 4oq8 and 4oq9 for STMV (Larson *et al.*, 2014). Data-collection and refinement statistics for crystals of thaumatin plus thioflavin and xylene cyanol can be found in Supplementary Tables S1 and S2. The structures of these complexes have been deposited in the PDB as entries 6c6w and 6e0d, respectively.

The structures of the crystals were refined using *REFMAC* (Murshudov *et al.*, 2011) or *CNS* (Brünger *et al.*, 1998) to an

R_{free} of less than 0.20 from known models as given above for all analyses to provide F_c.

3. Results

We first determined whether the retention of dyes by protein crystals was unique to lysozyme crystals or whether it was more general. We furthermore sought to evaluate the time periods over which the retention of dyes by crystals might be expected. To this end, we exposed crystals of lysozyme (tetragonal form), thaumatin and STMV to a variety of dyes. Sets of nine of the most highly colored crystals of lysozyme and thaumatin, and a set of 18 of the dyed STMV crystals, were then selected. Each sample in the sets was washed *in situ* several times with mother liquor lacking any dye and then placed in about 40 µl of clear mother liquor free of any dye or color. These were sealed and allowed to stand at room temperature, examined under a microscope daily, and then weekly, and eventually photographed.

Figs. 3, 4 and 5 show examples of the three kinds of crystals at day one and four months (120 d) later. With some exceptions, it is evident that even after standing for four months in dye-free mother liquor the crystals retained the dyes and remained colored, although in some instances the color was diminished. If the dyed crystals of each protein were collected and placed in dye-free mother liquor, then (as shown in Fig. 6) they continued to retain their color over a further 30 d, with each dyed crystal still being identifiable by its color. The experiment indicates that the retention of dyes is not confined to lysozyme crystals but is likely to be general. Indeed, a variety of other protein crystals were investigated, but less rigorously, and exhibited similar behavior. There were, however, exceptions in which protein crystals did not retain specific dyes, but these will be discussed in more detail elsewhere. The experiment further indicates that the dyes are

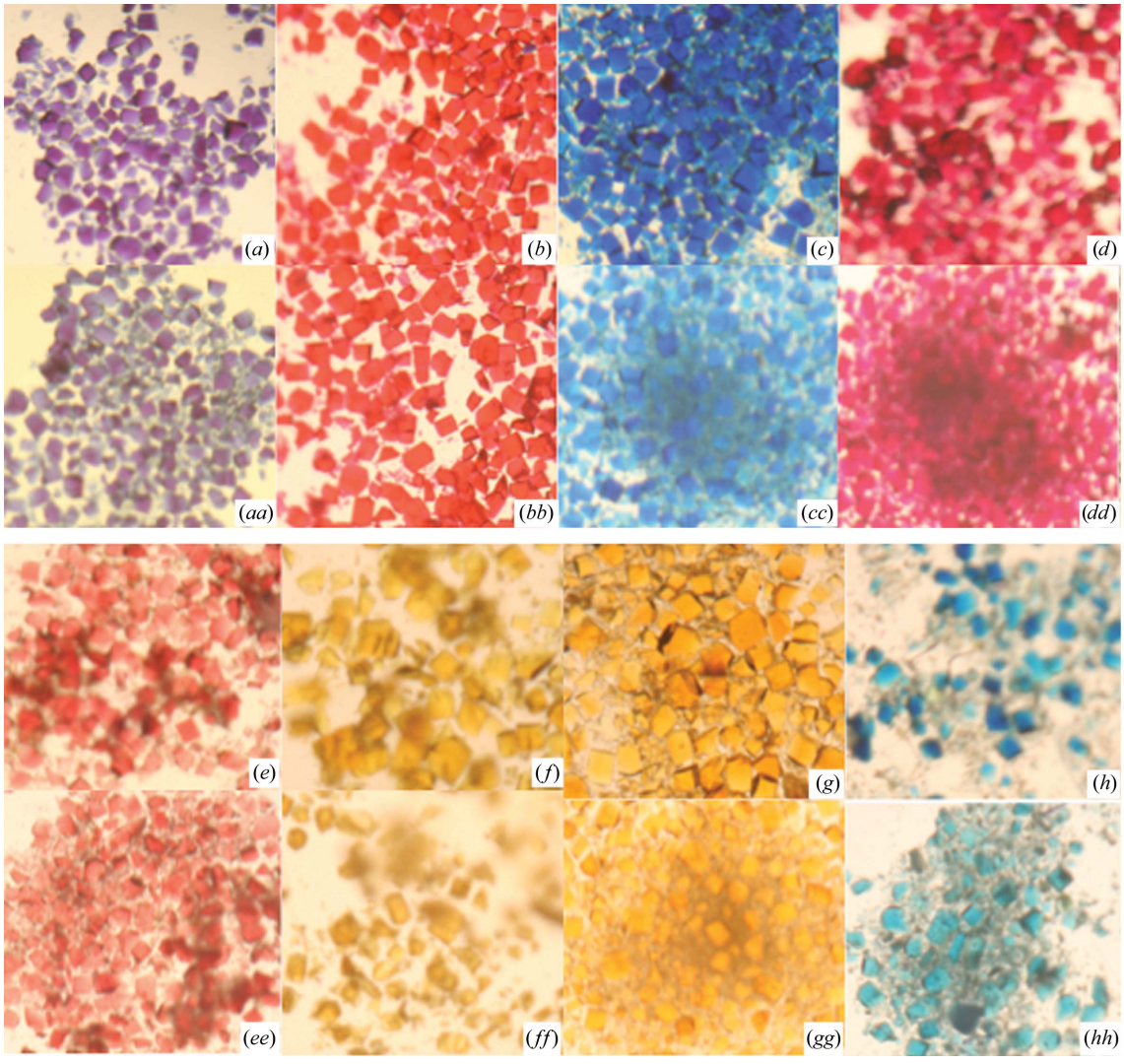


Figure 3
(*a*)–(*i*) show tetragonal lysozyme crystals thoroughly stained with (*a*) bromophenol blue, (*b*) erythrosine B, (*c*) xylene cyanol, (*d*) basic fuchsin, (*e*) chlorophenol red, (*f*) neutral red, (*g*) thioflavin T, (*h*) methyl orange and (*i*) methylene blue and photographed 24 h later. (*aa*)–(*ii*) show the same crystals, in the same order, photographed after 120 d in clear mother liquor. While the dyes in some samples have leached out of the crystals, most remain much the same in terms of color retention

firmly bound in the interior of the crystals and are retained almost indefinitely.

We wished to determine whether the dyes were likely to be retained in the crystals because the dyes were firmly bound to protein molecules, whether they were retained because they were trapped in lattice channels and interstices, or both. Microdialysis experiments were therefore carried out on nine different proteins, including a number of those studied here, to evaluate whether dyes were generally retained in dialysis cells by proteins in solution. The proteins were first exposed to saturating concentrations of dye and then dialyzed extensively, and the solution after 60 h of dialysis was examined visually to see whether it was still colored. Some samples were ambiguous, particularly for yellow dyes that were difficult to ascertain with assurance, but the results for most were clear. Table 3 presents the number of clearly colored samples after dialysis for each of the nine proteins. The retention of dye ranged from 100% of the dyes for bovine serum albumin to 41% for lysozyme. The results indicate that in many, but not all cases, the dyes are bound to the protein molecules themselves and are not simply retained in crystal-lattice spaces. Some dyes, however, that failed the microdialysis trials were nonetheless retained in crystal-incubation experiments. This indicates that

Table 3
Retention of dyes by dialyzed proteins.

Protein	No. of dyes bound	% Bound
Bovine serum albumin	19/19	100
Human gamma globulin	17/21	81
Egg albumin	14/19	74
Jack bean canavalin	15/21	71
Porcine pepsin	13/19	68
Thaumatin	12/22	55
Hen egg-white lysozyme	8/18	44
Concanavalin A	8/19	42

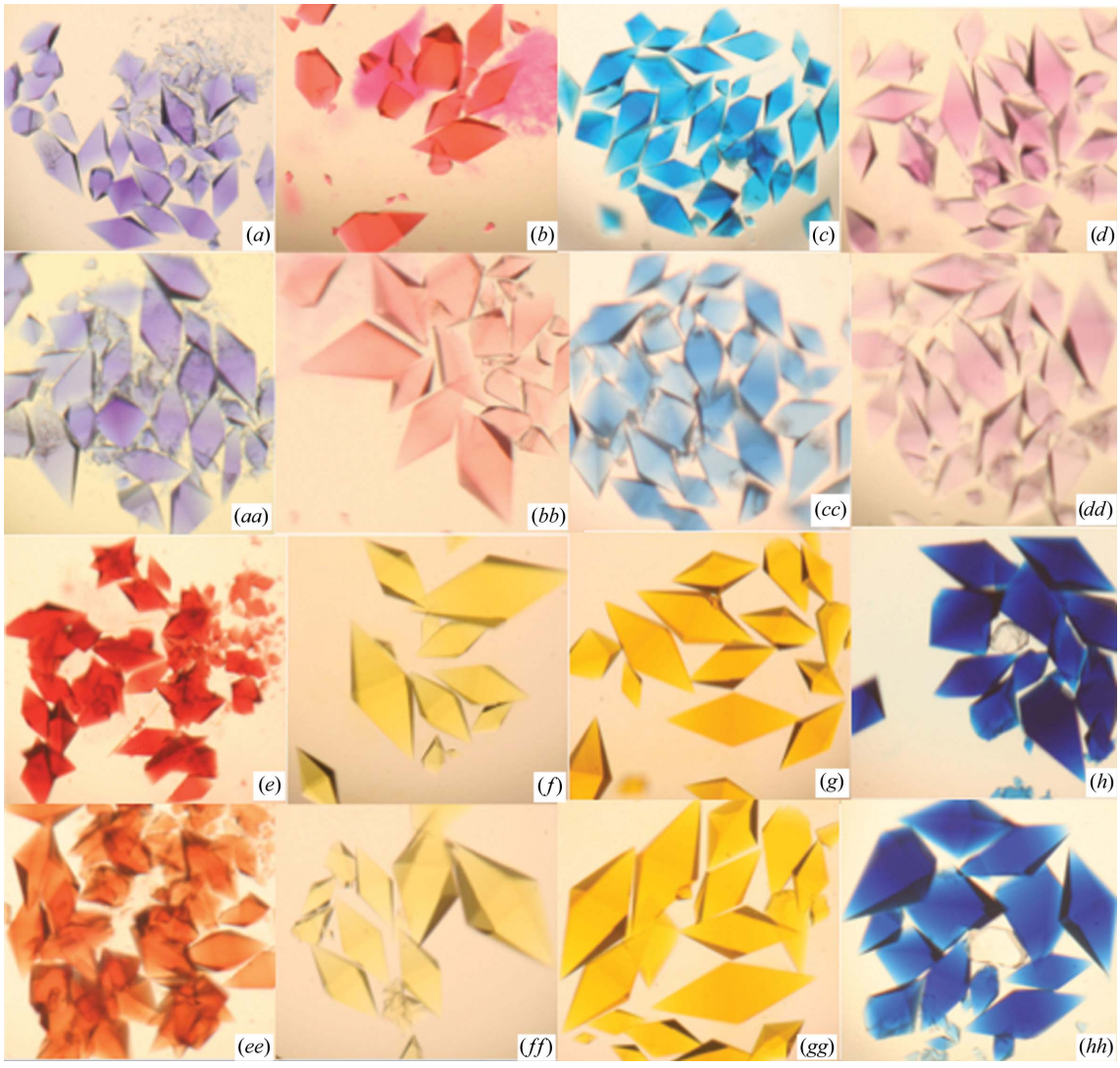


Figure 4
(*a*)–(*h*) show tetragonal thaumatin crystals thoroughly stained with (*a*) bromophenol blue, (*b*) erythrosine B, (*c*) xylene cyanol, (*d*) chlorophenol red, (*e*) neutral red, (*f*) thioflavin T, (*g*) methyl orange and (*h*) methylene blue and photographed 24 h later. (*aa*)–(*hh*) show the same crystals, in the same order, photographed after 120 d in clear mother liquor. While the dyes in a few samples have leached out of the crystals, most retain their original stained color.

crystals may also have retention properties by the creation of binding sites.

For lysozyme, the colored protein solutions remaining after extensive dialysis were set up for crystallization. Most crystallized, and as can be seen in Fig. 7 the crystals were colored according to the dye retained by the protein molecules. This demonstrates that the protein molecules bind the dye, retain the dye through dialysis and carry the dye into the lattice when they crystallize. This would additionally imply that the dyes are tightly bound to the protein molecules. It further indicates that the dyes do not prohibit crystallization in spite of their binding.

The final question that we sought to answer was how the dyes are bound in the crystals. There appear to be four possibilities: (i) the dyes are closely associated with the protein molecules and are bound by protein–dye interactions (as suggested by the above experiments); (ii) the dyes are trapped in spaces and channels within the crystals created by the lattice (also consistent with the above experiments); (iii) both (i) and (ii); and (iv) in those cases where the dye is not significantly retained by specific protein crystals, none of the previous choices. To gain some insight into the question, X-ray analyses using difference Fourier methods were applied to the protein–dye crystal complexes listed in Table 2, all of which were richly colored at the time of data collection.

The results are fairly easy to summarize. Difference Fourier maps, $F_o^{dye} - F_o^{nat}$, $F_o^{dye} - F_c^{nat}$ and $2F_o^{dye} - F_c^{nat}$, revealed no difference density that would indicate the presence of dye for lysozyme, trypsin and STMV crystals and for almost all of the thaumatin crystals. There was no evidence of specific binding to the protein molecules or ordered molecules of the dye in solvent interstices.

As exceptions prove the rule, dyes were found in some thaumatin crystals which were co-crystallized with the dyes. In the thaumatin–xylene cyanol crystals, prominent density (as seen in Fig. 8) was found exactly on a crystallographic twofold

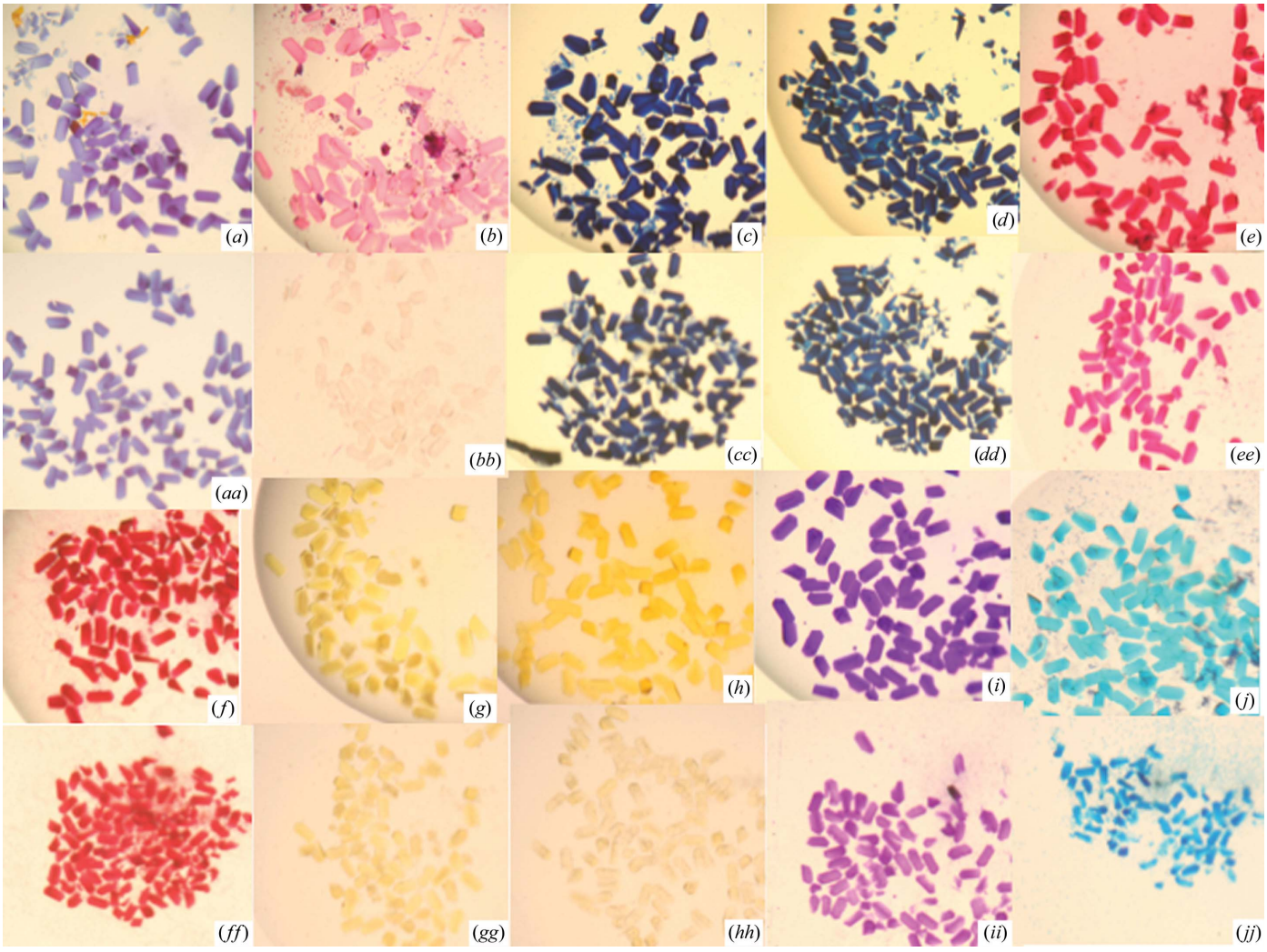


Figure 5
(*a*)–(*j*) show orthorhombic satellite tobacco mosaic virus crystals thoroughly stained with (*a*) bromophenol blue, (*b*) chlorophenol red, (*c*) xylene cyanol, (*d*) Evans blue, (*e*) basic fuchsin, (*f*) neutral red, (*g*) thioflavin T, (*h*) methyl orange, (*i*) crystal violet and (*j*) *m*-cresol purple and photographed 24 h later. (*aa*)–(*jj*) show the same crystals, in the same order, photographed after 120 d in clear mother liquor. Almost all of the samples remain much the same in terms of color retention.

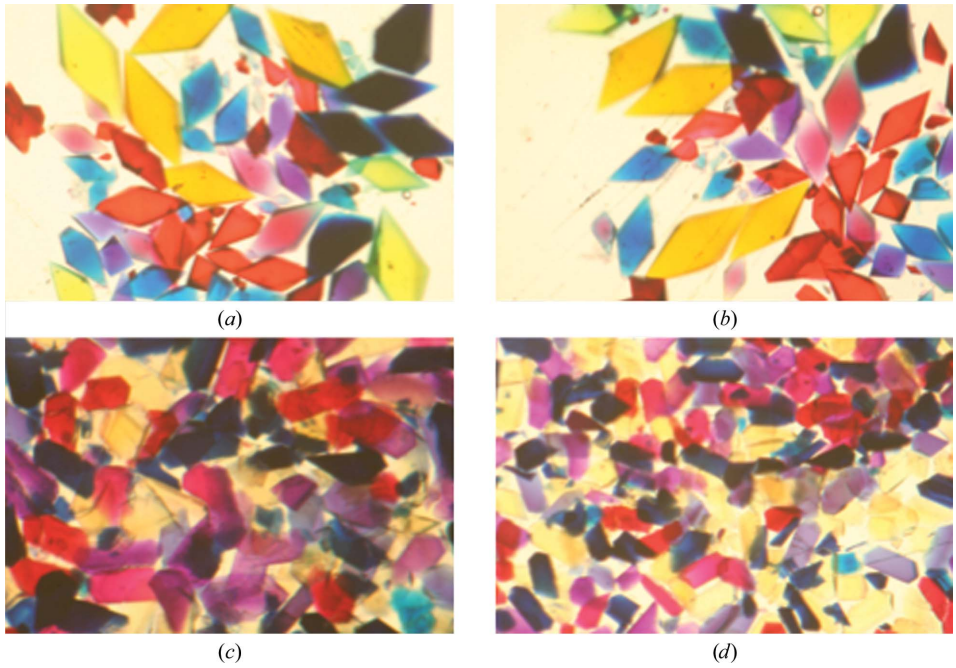


Figure 6
(*a*) and (*b*) show tetragonal thaumatin crystals that were stained with a variety of dyes, removed from their individual stains, washed with mother liquor and placed in a large volume of stain-free mother liquor with others. Photographs were taken 5 d later. Crystals stained with specific dyes are clearly recognizable by their characteristic colors. (*c*) and (*d*) show orthorhombic satellite tobacco mosaic virus crystals that have been similarly treated.

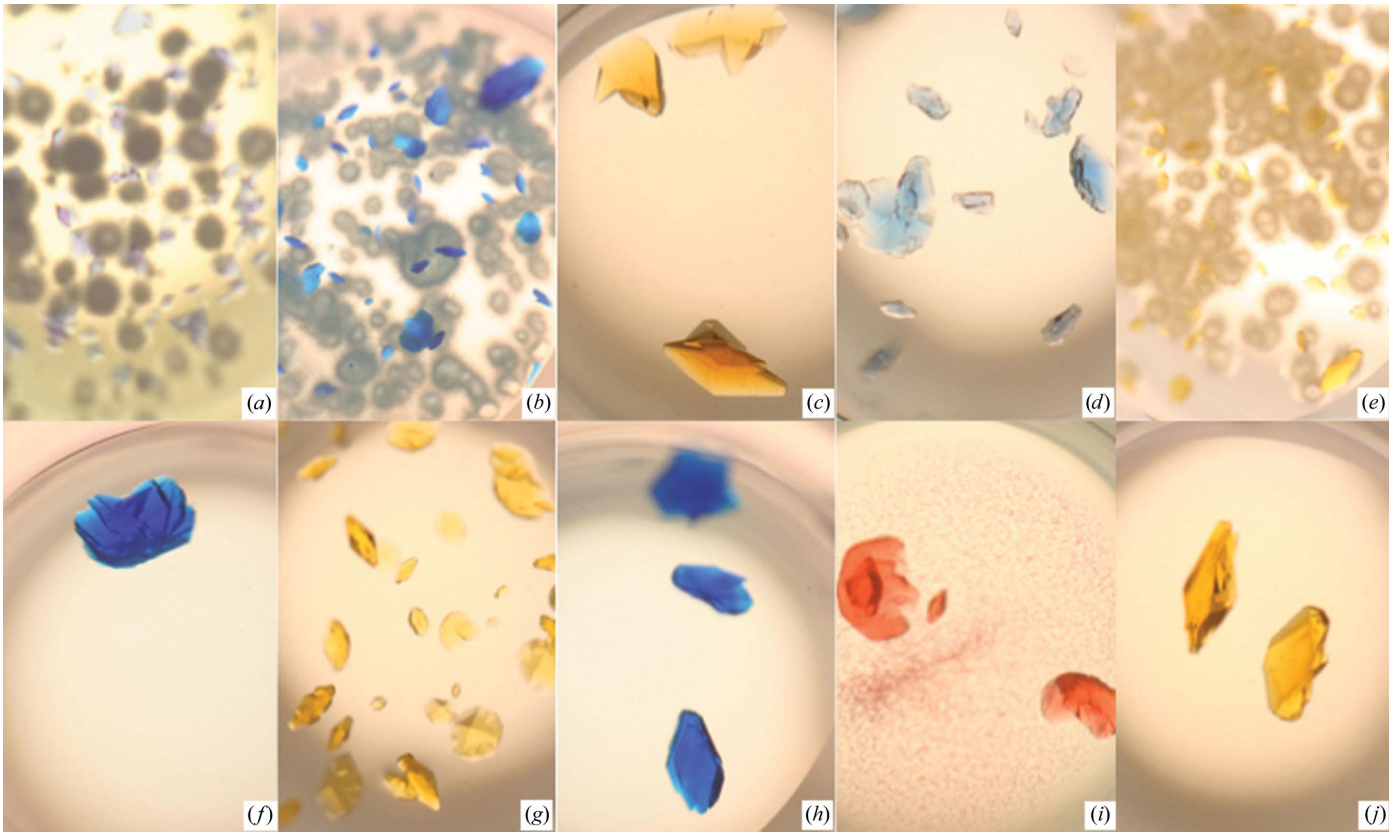


Figure 7
Lysozyme protein was exposed in solution for 24 h to an excess of stain and thoroughly dialyzed, and those samples that retained the dye color were then set up for crystallization. Most of these lysozyme samples crystallized, and the resultant crystals exhibited the color of the stain to which they were originally exposed. The crystals shown here were exposed as protein in solution to (*a*) bromophenol blue, (*b*) xylene cyanol, (*c*) methyl orange, (*d*) *m*-cresol purple, (*e*) chlorophenol red, (*f*) Evans blue, (*g*) thioflavin T, (*h*) methylene blue, (*i*) erythrosine B and (*j*) neutral red.

axis consistent with a single molecule of xylene cyanol that was twofold disordered. That is, it entered the lattice in either of two dyad-equivalent orientations. Similar density was found in difference maps for crystals of thaumatin complexed with *m*-cresol purple, which has a structure somewhat comparable to xylene cyanol that was twofold disordered (PDB entry 6eod).

Another result (see Supporting Information) in which density appeared was for crystals of thaumatin complexed with the dye thioflavin T, a dye that is commonly used to stain for amyloid proteins (Biancalana & Koide, 2010). Again, prominent density was located on a crystallographic twofold axis at a position different from that for xylene cyanol, but in this case the density could readily be fitted by two thioflavin T molecules (PDB entry 6c6w). The two molecules were twofold-related and tightly stacked on one another about the dyad. These are shown in Fig. 9. This thioflavin T stacked dimer is almost identical to a thioflavin T dimer previously observed bound to the protein microglobulin (Halabelian *et al.*, 2015). The examples of xylene cyanol, *m*-cresol purple and thioflavin T demonstrated that if dye molecules were present and ordered in the crystals that we investigated then our methods were adequate to reveal them. In all cases involving xylene cyanol, *m*-cresol purple and thioflavin T, the ordered dyes observed in the difference Fourier maps were insufficient to alone explain the intense color of their respective crystals.

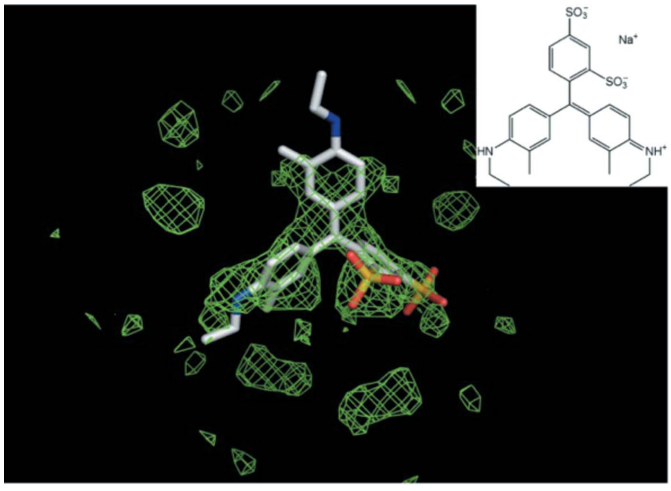


Figure 8
Difference electron density is shown superimposed on a molecule of xylene cyanol that lies exactly on a crystallographic twofold axis at a position different from that where the thioflavin T dimer is bound. The xylene cyanol is twofold disordered and assumes one of two dyad-related orientations at any binding site.

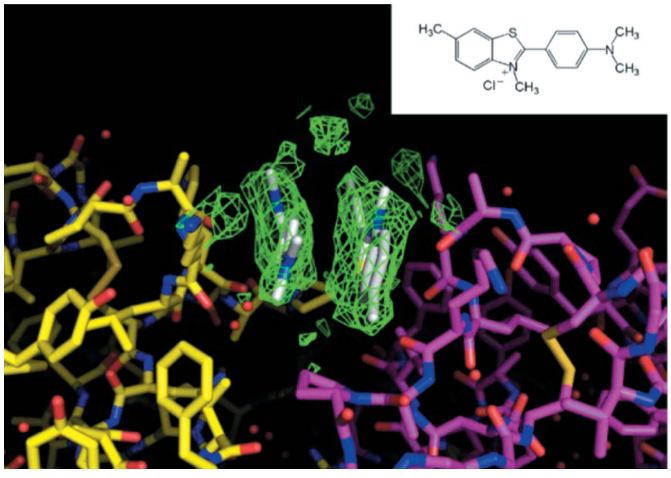


Figure 9
Difference electron density is shown superimposed on a dimer of the dye thioflavin T bound to the protein thaumatin. The two monomers of the dimer are dyad related by a crystallographic twofold axis that passes between them. The thioflavin T dimer is bound at an interface between twofold-related thaumatin molecules in the lattice.

4. Discussion

The results from these experiments represent something of a paradox. The dye-retention experiments with lysozyme, thaumatin and STMV, the dialysis experiments with nine proteins and the crystallization experiments with dialyzed dye–lysozyme samples all indicate that the dyes are firmly bound within the protein crystals, if not to the free protein molecules themselves. On the other hand, X-ray diffraction analyses that should reveal the presence of any ordered material in the crystals show, with the exception of the thaumatin cases noted, that there is nothing to be seen. This is true for four different macromolecular crystals and for a diverse set of common biological stains.

The trivial answer, of course, is that the dye molecules are in the crystals, as they must be since the crystals are richly colored, but that the dye molecules are sufficiently disordered that they are invisible to X-ray diffraction analysis. The difficulty with this easy answer is that it does not explain how completely disordered molecules can, at the same time, be tightly bound by the protein molecules and by the crystal lattice. Enzymes, for example, which have affinities for substrates and coenzymes that are probably in the same range of affinities as for these dyes, do not bind disordered substrates and coenzymes, but exquisitely ordered molecules (Fischer, 1890). How, then, can we observe relatively high affinity between proteins and completely disordered conventional molecules?

The most attractive hypothesis that comes to mind (indeed, there may be others) is based on analogy with another molecular structural combination with which we are familiar; that is, detergent–membrane protein complex crystals. In these crystals detergent makes up a very large proportion of the crystal (DeLucas, 2009; Loll, 2003; Michel, 1991; Wiener, 2004; Zulauf, 1990) and more or less fills interstitial spaces along with solvent. The detergent, however, is never, or at least seldom, visualized in electron-density maps. The dyes, with their large hydrophobic cores and solubilizing hydrophilic groups, may be acting as detergents that surround exposed hydrophobic patches on the exterior of protein molecules. Indeed, it is well known that detergents that are bound in this way are often difficult to remove or replace even by extensive dialysis (DeLucas, 2009; Loll, 2003; Michel, 1991; Wiener, 2004; Zulauf, 1990), as are the dyes. In addition,

'lobster-trap' intersticies and crevices in the crystal lattice, which allow easy entry but are difficult to escape, may also contribute.

A second possibility, which is somewhat similar to the first but less exact, is that the dyes act like a molecular grease that coats certain areas on the proteins. The interactions are imprecise and nonspecific, but nevertheless firm in an aqueous environment. One can smear grease on a glass plate and submerge it for months in water, even with agitation, and upon removal from the water the grease will still be found smeared on the plate. We concede that these hypotheses are speculative, but seem consistent with the available evidence.

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