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Penetration of dyes into protein crystals

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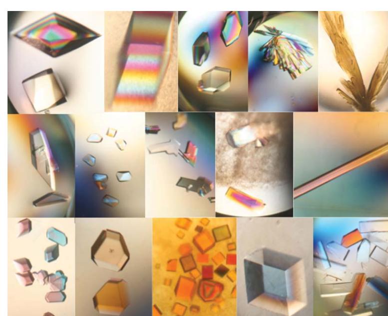
Experiments were carried out on 15 different protein crystals with the objective of estimating the rates of penetration of dye molecules into the crystals. The dyes were in the molecular-weight range 250–1000 Da and the protein crystals were of dimensions of 0.7 mm or greater. Experiments were also conducted on protein crystals grown between glass cover slips (separation 200 µm) that restricted the direction of diffusion. The rate of penetration of dyes into protein crystals depends very much on the degree of association between the dye and protein molecules. Dye penetration was not consistent with pure diffusion when the affinity of the protein for the dye was significant, and this was frequent. Penetration rates were less dependent on factors such as the molecular weight of the dye or the diffusion direction. For weakly interacting protein crystal/dye combinations, penetration was a fair measure of diffusivity and the observed rates were in the range 60–100 µm h^{-1}. For strongly interacting combinations, the rates of penetration were of the order of 15–30 µm h^{-1}.

1. Introduction

Crystalline complexes of proteins with conventional small molecules and ions have many applications, and are of great value in macromolecular crystallography (McPherson, 1992, 1994). Complexes of enzymes with coenzymes, substrates and inhibitors are classical paths to understanding enzyme specificity, mechanisms and catalysis (McPherson *et al.*, 1987; McPherson, 1994; Petsko & Ringe, 2004). The design of drugs and pharmaceuticals, and fragment screening are dependent on visualizing the molecules bound to the target protein in the crystalline state. Phase determination by isomorphous replacement, and in many instances by anomalous scattering approaches, is often dependent on the derivatization of crystalline proteins by the diffusion of electron-rich atoms into the crystal structure (Blundell & Johnson, 1976; Rupp, 2010). Finally, modern cryo-crystallography involves submerging crystals, usually briefly, into a cryoprotective agent, which is a concoction of small organic molecules (Pflugrath, 2004, 2015; Garman & Schneider, 1997).

1.1. Objectives

A question that arises is: at what rate do small molecules and ions diffuse through protein crystals, and what period of time is required for a protein crystal to become saturated? These are challenging to address quantitatively because they involve many factors and no general answer is likely to exist. By investigating a number of examples, however, we can at least acquire some idea of the likely ranges. This was the intention of the experiments described here. The objectives were modest, but they have the virtue of being realistic.



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1.2. Protein–dye interactions affect penetration

A critical consideration in terms of entry and exit rates is whether the small molecule associates significantly with the protein molecules comprising the crystal, whether and how much it is retarded by the interactions, and whether it is bound and immobilized as it passes through the structure. This is more common than might be suspected. As suggested elsewhere, dye molecules probably associate with protein molecules through a detergent-like mechanism (McPherson & Larson, 2018). If no significant retardation occurs, then it is a matter of the simple diffusion of a small molecule through a porous material. If significant associative interactions occur, then the progress of small molecules through the crystal is not ruled by diffusion alone but is, in addition, dependent on how strongly they interact with the protein.

To measure the pure diffusion rates of small molecules through a crystal, small molecule/crystal combinations that experience no appreciable small molecule–protein interactions must be used. Such combinations are evident when the crystals, submerged in a dye containing mother liquor, take on an intensity of color that is no greater than that of the surrounding liquid. That is, they do not concentrate the dye. More reliably, such cases can be recognized when the diffusion rate into the crystals is approximately equal to the rate of diffusion out of the crystals and the return to clarity. If the latter condition does not hold, then the small molecule will be retained and the diffusion out of the crystal will be slower or may not occur at all (the latter is discussed in more detail in McPherson & Larson, 2018).

1.3. What is penetration and how was it measured?

A straightforward quantity to determine experimentally is the time required to saturate a crystal with the small molecule. This requires only that the saturation point be evident and unambiguous: it is the time at which the protein crystal attains maximum color. The time period for saturation may be more useful to the investigator as it provides a guide to the length of time that a crystal must be exposed to a conventional molecule before it is saturated. In the experiments presented here, we use dyes that exhibit intense colors in the visible spectrum. This allowed direct visual measurement of the rates at which they pass into and out of various protein crystals, and to assess when the protein crystal becomes saturated with the dye.

1.4. The dye molecules

Most biologically useful dye molecules (Horobin & Kiernan, 2002; Gurr, 1971) have aromatic, conjugated, hydrophobic cores that serve as the chromophore and have attached, solubilizing, hydrophilic groups that carry either a positive charge (basic dyes) or a negative charge (acidic dyes). In these experiments, the dyes spanned a range of molecular weights from about 250 Da to about 1000 Da, with most in the range 300–500 Da. The dyes investigated are listed in Supplementary Table S1. It is worth noting that the molecular-weight range is comparable to that of many coenzymes and prosthetic groups employed in crystallographic studies. In addition, the dyes, which have aromatic moieties covalently attached to solubilizing electrostatic groups, are somewhat similar to many of these same molecules, such as nucleotide coenzymes and substrates.

Investigations were carried out on a number of different protein crystals and these are shown in Fig. 1. The most extensive experiments involved tetragonal and monoclinic crystals of lysozyme, thaumatin, trypsin inhibited with benzamidine, and glucose isomerase, and rhombohedral and orthorhombic crystals of canavalin, concanavalin A, Satellite tobacco mosaic virus (STMV), concanavalin B, RNase S and β-lactoglobulin. Because it was necessary to follow the movement of the dyes through the crystals over an extended period, and to estimate their saturation points using a medium-magnification light microscope, the crystals had to be relatively large. The crystals we used therefore had dimensions of about 0.7–1.5 mm on an edge.

2. Experimental

Crystallization conditions for the proteins investigated can be found in Supplementary Table S2, which describes the conditions derived during this work, and often do not follow literature precedent. The crystallographic properties and solvent volumes of these crystal forms are reported in Supplementary Table S3. Crystals were grown by vapor diffusion in sitting-drop Cryschem plates with drop sizes of 8–12 µl and reservoirs of 0.6 ml. All crystallizations were carried out at 22°C, although the plates were moved to 4°C to better preserve the crystals once they had grown. The mother liquors of canavalin crystals were made 15% in MPD after the crystals had fully grown as canavalin crystals may degrade spontaneously in vapor-diffusion settings over a week following their appearance.

Manipulation of the mother liquors in the droplets was conducted under observation by a zoom-lens dissecting microscope using gel-loading tips on 10 µl pipettes. Mother liquor was drawn away using paper wicks (Hampton Research, Aliso Viejo, California, USA). Any necessary manipulation of the crystals, such as transfer to alternate mother liquors, was performed using cryo-mounting tips of 50–300 µm in diameter from MiTeGen (Ithaca, New York, USA). The observation of diffusion experiments used an SZX12 Olympus zoom microscope with an adaptor for a Canon EOS digital camera. Photographs were taken with the night/no-flash setting. Pairs of photographs were recorded at timed intervals at high and low magnification. Observation and photographic recording took place over a period of at least 24–48 h and of up to several days when the penetration of dye was very slow.

A crystal-perfusion experiment normally proceeded in the following manner. A drop of mother liquor containing one or more large crystals was selected along with a dye. Dyes that were blue, red or purple were preferred because they could be best visualized, although experiments were also carried out with yellow dyes (thioflavin T and acriflavin). Mother liquor having the same chemical composition as the mother liquor in the droplet, but with no protein component, was made a rich

color by addition of the dye, usually requiring only 1–2 µl of the concentrated (often saturated) dye. Under the microscope, the original, clear mother liquor was drawn away to near-dryness with a micropipette and wick. 2–5 µl of the mother liquor containing the dye was immediately added so that the crystal(s) was surrounded with color. It was necessary to refresh the dye around the crystal when the crystal absorbed a large amount of the dye and the surrounding mother liquor paled in color.

For large crystals, dye was added so that one or more faces of the crystal were not in direct contact with the dye-containing mother liquor. This made it easier to follow the penetration of the dye into the crystal. This could be achieved when a crystal terminated at the sitting-drop surface and the top face was unobscured. The top face essentially provided a window that allowed observation of the crystal at its center. When the crystal grew at the very edge of its droplet, dye entered from only one or two sides and again a surface was not exposed. When a crystal face could not be isolated for viewing, dye diffused into the crystal isometrically, still allowing overall color change and the time to saturation to be recorded. All perfusion experiments were carried out at 22°C.

Another approach employed was to grow protein crystals between two glass cover slips with a separation distance of 200 µm. In these sandwich arrangements the crystals completely filled the space between the slides. When exposed to dyes injected into the mother liquor around them, diffusion occurred two-dimensionally from the edges of the crystals inwards, with no penetration from the faces of the crystals limited by the glass slides. These experiments provided an unimpeded observation of the movement of dyes through the crystals with no confusion regarding directionality.

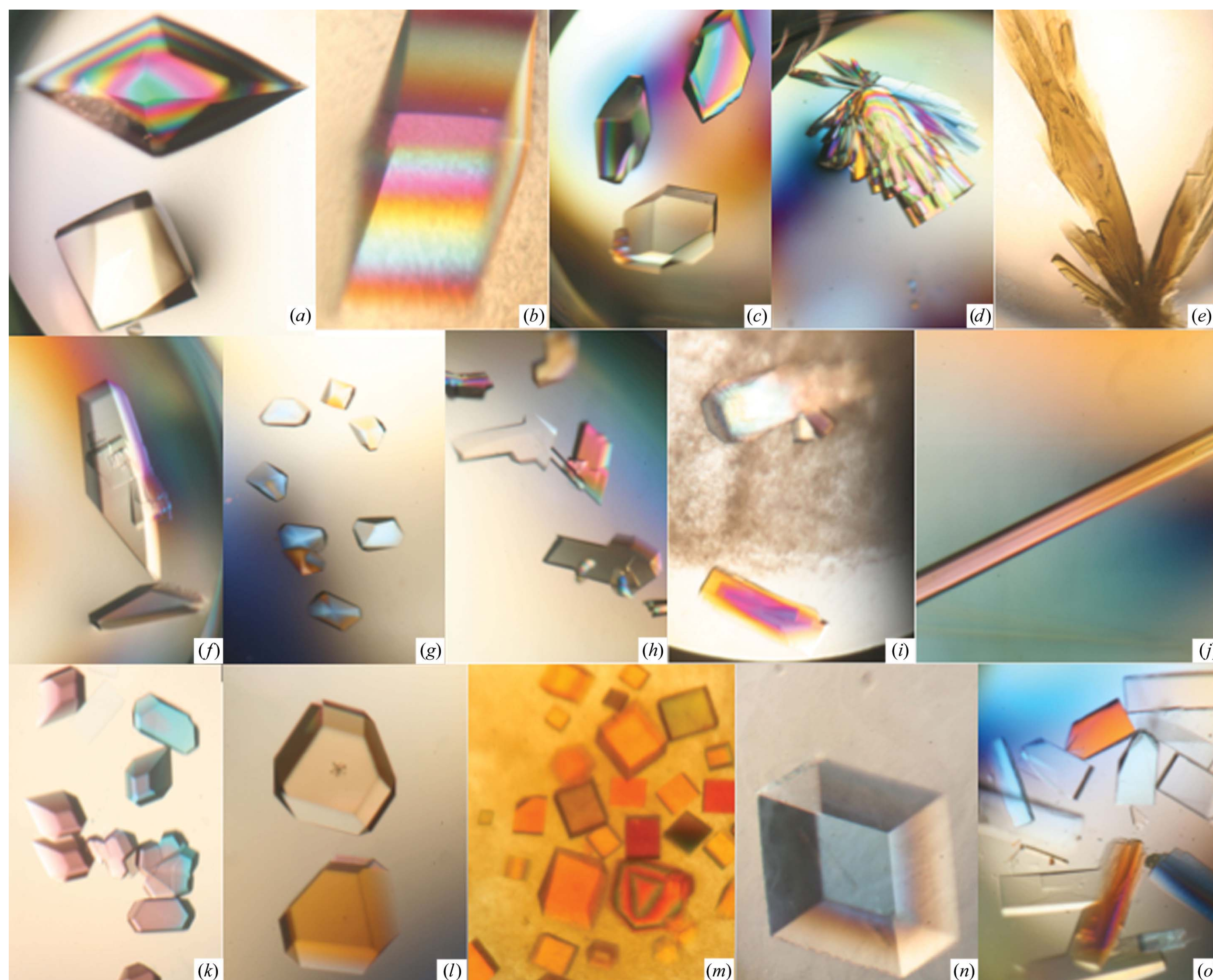

Figure 1
The protein crystals used in this investigation are shown. They are (*a*) thaumatin; (*b*) bovine trypsin inhibited with benzamidine; (*c*) tetragonal hen egg-white lysozyme; (*d*) monoclinic hen egg-white lysozyme; (*e*) orthorhombic beef catalase; (*f*), (*g*), (*h*) bovine ribonuclease S; (*i*) bovine β-lactoglobulin; (*j*) concanavalin B; (*k*) Satellite tobacco mosaic virus; (*l*) bacterial glucose isomerase; (*m*) concanavalin A; (*n*) rhombohedral canavalin and (*o*) orthorhombic canavalin.

3. Results

3.1. Entry and exit of dyes into/from crystals

It is necessary to discriminate four classes of penetration processes depending on the interactions of the individual dyes with specific protein crystals: color change of the crystal as a function of time (i) when the dye associates significantly with the protein in the crystal and is either retarded or bound by the protein molecules; (ii) when the dye enters into traps in the crystal, formed by interstitial spaces in the structure, from which it cannot escape easily or at all; (iii) both cases (i) and (ii) above; and (iv) when the dye does not interact strongly with the protein, is not significantly retarded and is not subject to trapping.

Cases (i), (ii) and (iii) are difficult to resolve in most instances, although our results suggest that (ii) is likely to be of minor significance. Case (iv), however, can be readily identified because the rate of diffusion out of the crystal and the return to clarity, when placed in dye-free mother liquor, is approximately the same as the rate of diffusion into the crystal. The crystal is essentially a porous material penetrated by channels of periodic structure and the dye diffuses freely in and out. These cases allowed us to measure the true rate of dye diffusion through crystalline material in the absence of significant protein–dye interactions.

Orthorhombic and rhombohedral canavalin crystals were particularly useful in this regard as they readily accepted dye diffusing in from the exterior but returned to clarity when submerged in dye-free mother liquor. Lysozyme crystals also showed this feature with non-interacting, nontrapping dyes, while showing a completely different behavior with other dyes. Thus, two fundamentally different crystal/dye combinations pertain. One is when free diffusion prevails, and the other is combinations for which diffusion is retarded by protein and/or lattice interactions. For the first case the penetration rates are essentially diffusion rates, while for the remainder, penetration rates and saturation times are the only meaningful measures.

Figs. 2, 3, 4 and 5 show orthorhombic canavalin crystals staining with methylene blue, bromophenol blue, xylene cyanol and erythrosine B, respectively. The last few frames in Figs. 3–5 demonstrate the return to clarity after the crystals were placed in dye-free mother liquor and the dye diffused away. The diffusion of the dye out of the crystals and the return to clarity for these experiments was comparable to the rate of saturation of the crystals.

After saturation with dye, as seen for crystals of glucose isomerase and thaumatin staining with the dye basic fuchsin in Figs. 6 and 7, many crystals retained their full intense color after being submerged in a large volume of dye-free mother liquor even for months. It was evident that a strong association between the specific dye and the protein was present in these crystals, and therefore no release of the dye from the crystal occurred. On the other hand, in Fig. 5 back-diffusion in dye-free mother liquor did not return the crystal to complete clarity, but to a very faded or weakly stained condition. For these combinations, it was probable that only weak association occurred between the dye and the protein, and penetration approximated the pure diffusion examples.

Combinations of individual dyes with protein crystals such as those seen in Figs. 6 and 7, in which strong protein–dye interactions pertained, were common. Penetration of dyes into

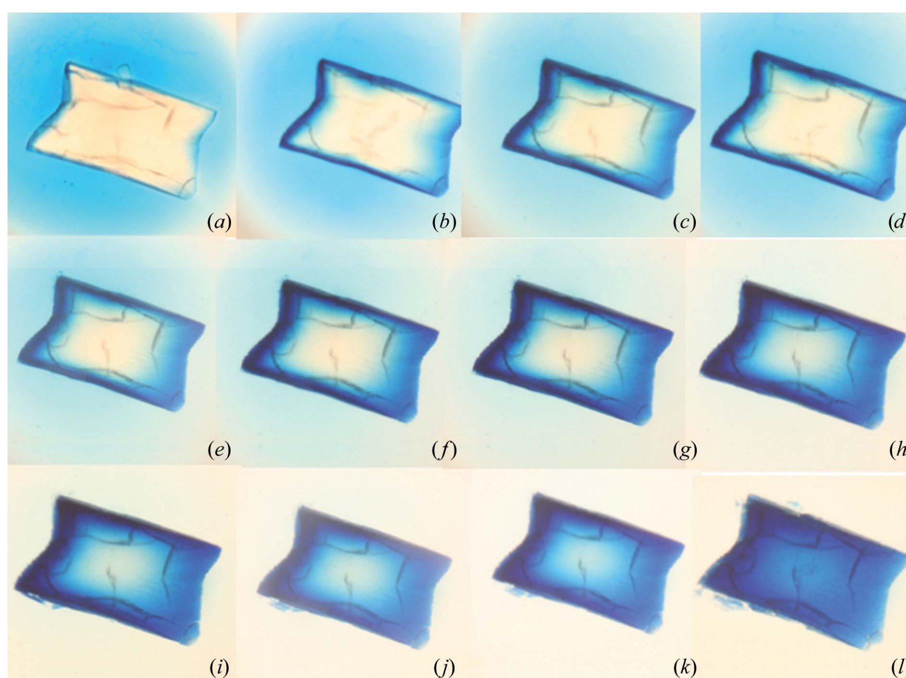

Figure 2
Canavalin perfused with methylene blue. An orthorhombic canavalin crystal, about 1.5 mm in the longest dimension, was exposed to the dye methylene blue by direct addition of the dye to the mother liquor. Photographs (*a*) to (*l*) were then taken at intervals over a period of about 18 h. By the end of this period the crystal was essentially saturated with the dye.

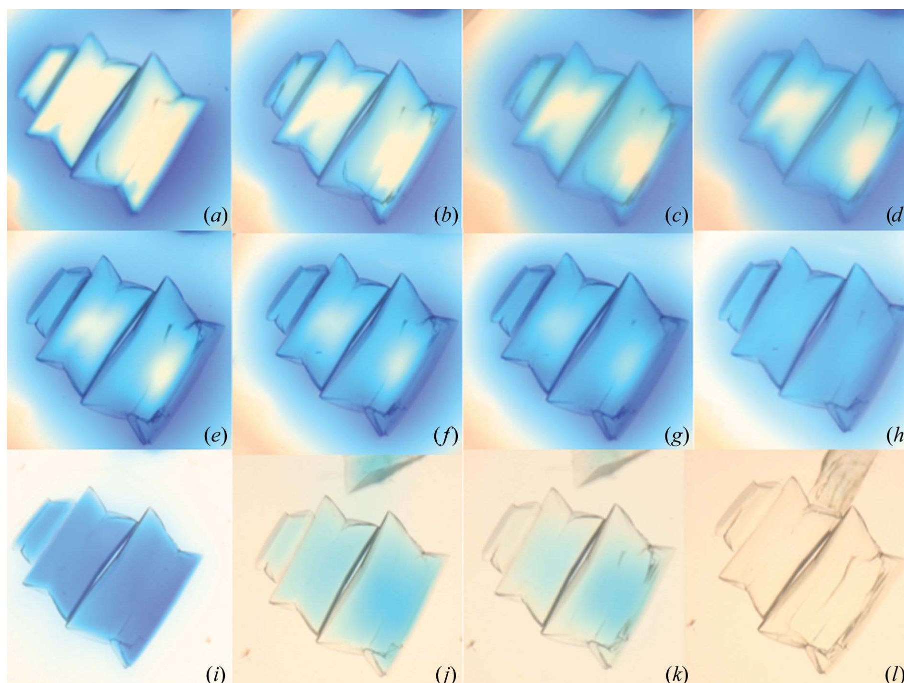

Figure 3
Canavalin perfused with bromophenol blue. Orthorhombic crystals of canavalin of 1.5 mm in length were exposed to mother liquor containing the dye bromophenol blue and photographed (*a*–*i*) over a period of about 20 h. The crystals, once saturated with dye (*i*), were then placed in dye-free mother liquor. The dye diffused out of the crystals (*j*–*l*), returning them to clarity (*l*) in about 18 h. (*j*) is after 6 h, (*k*) after 12 h and (*l*) after 18 h. This is an example of pure diffusion of dye through a crystalline protein where the dye experiences no strong association with the protein or is trapped by the structure.

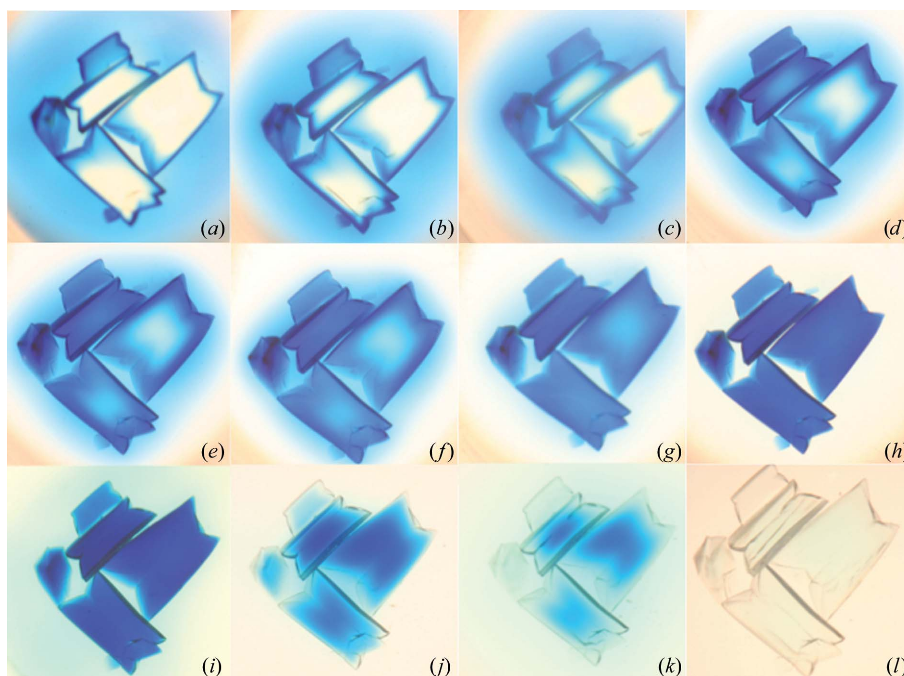

Figure 4
Canavalin perfused with xylene cyanol. A series of photographs (*a*–*i*) that follow the movement of the dye xylene cyanol through large orthorhombic crystals (1.5 mm in length) of the protein canavalin. The total time from (*a*) to (*i*) was about 19 h. The crystals in (*i*), saturated with methylene blue, were then placed in dye-free mother liquor and images (*j*) to (*l*) follow destaining to clarity over a period of again about 19 h. (*j*) is after 3 h, (*k*) after 7 h and (*l*) after 19 h.

crystals where diffusion out was negligible was much slower than for free diffusion. Additional examples are shown in Figs. 8 and 9 for crystals of concanavalin A plus crystal violet and thaumatin plus xylene cyanol, respectively. For these crystals the progress of the dye diffusing into the crystals was continuously retarded by the binding, immobilization and removal of dye molecules from the diffusion front. For these dye/crystal combinations the penetration rates depended more profoundly on the extent of interaction of the dye with the protein than on other properties such as the molecular

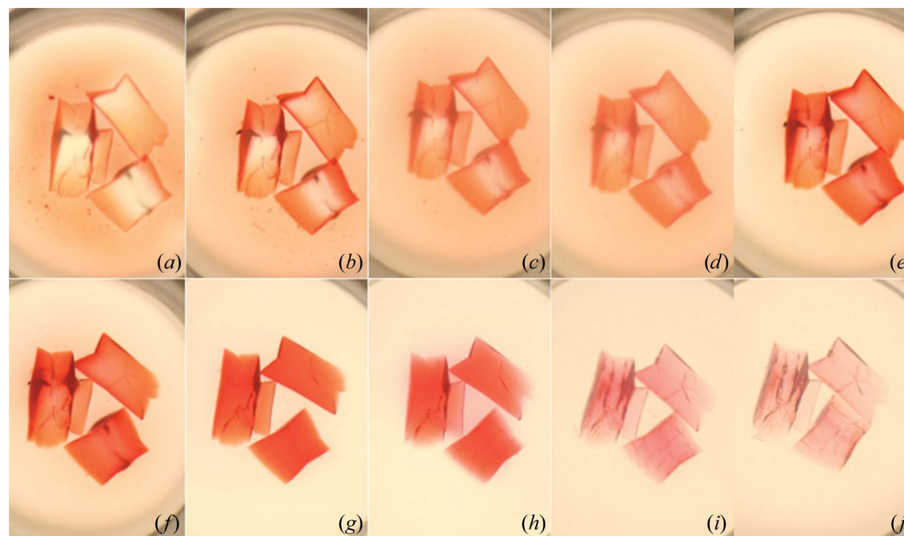

Figure 5
Canavalin perfused with erythrosine B. Images (*a*) to (*f*) follow the staining of orthorhombic canavalin crystals of maximum dimension about 1.2 mm with the dye erythrosine B over a period of about 22 h. The crystals, after replacement of the mother liquor with an equivalent dye-free mother liquor, are seen to destain in (*g*) to (*j*) over a period roughly the same as that for the staining process to complete. (*h*) is after 3 h, (*i*) after 10 h and (*j*) after 18 h.

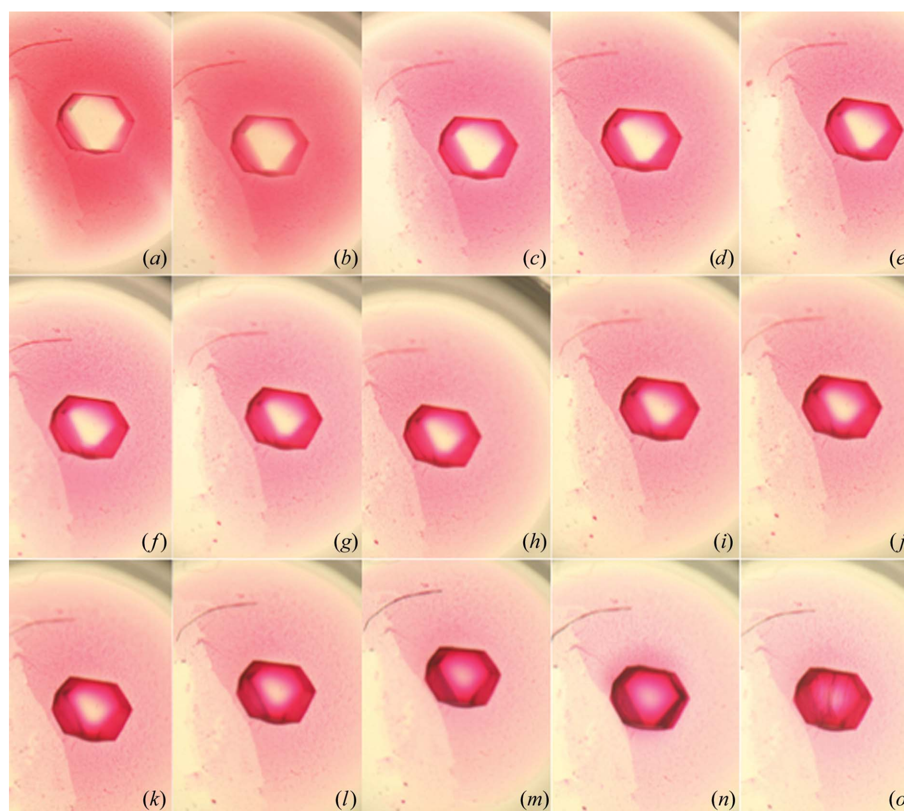

Figure 6
Glucose isomerase perfused with basic fuchsin. An orthorhombic crystal of bacterial glucose isomerase of about 0.85 mm in diameter was exposed to the dye basic fuchsin directly added to its mother liquor. Images (*a*) to (*o*) record the incorporation of the dye over a period of about 42 h. When the saturated crystals were placed in dye-free mother liquor they indefinitely retained the dye.

weight and concentration of the dye, variables that would significantly affect pure diffusion.

For dye/crystal pairs where the interaction between protein and a specific dye was insignificant, the saturation time of dyes for a large crystal was 18–22 h, yielding diffusion rates through the crystals of about 60–100 µm h^{-1} for dyes in the molecular-weight range 300–600 Da, with an average of about 75 µm h^{-1}. This estimate is based principally on experiments involving tetragonal crystals of lysozyme, concanavalin A and both rhombohedral and orthorhombic crystals of canavalin. Within all of the crystals where strong interactions occurred between a specific dye and a protein, the range was wider and the penetration rates were much lower. For the examples shown in Figs. 6–10, the time required for the dye to reach the center of a crystal or to saturate a crystal was of the order of 48 h.

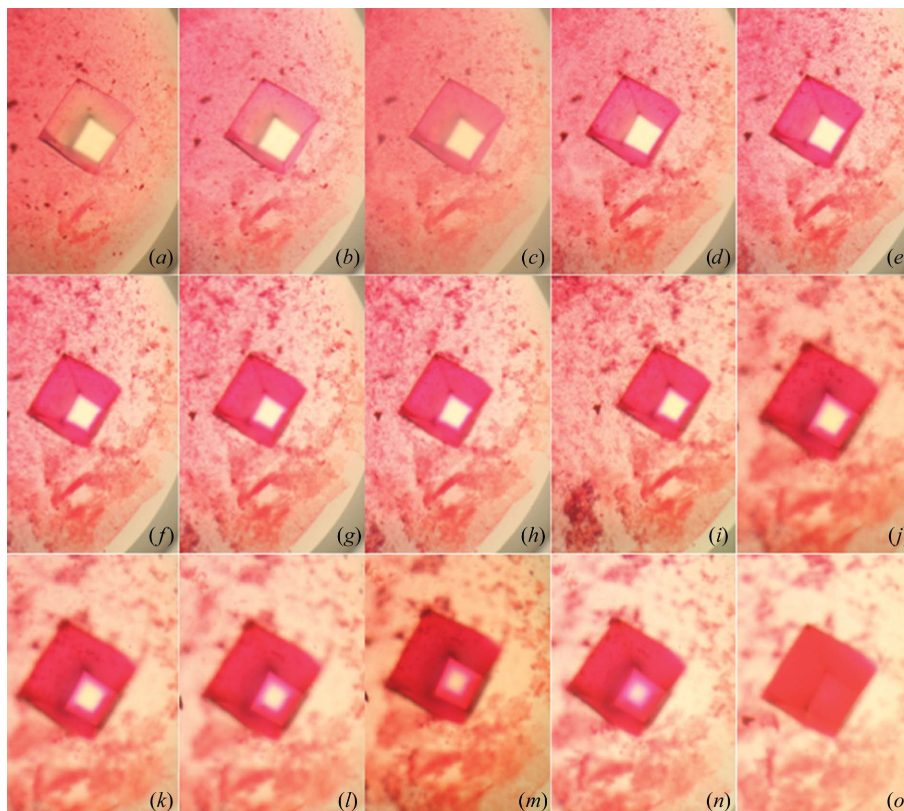

Figure 7
Thaumatin perfused with basic fuchsin. A tetragonal crystal of thaumatin is shown as it acquires the dye basic fuchsin from its mother liquor. The total time to saturation was about 47 h. The view here is approximately along the fourfold axis. The crystal is about 0.6 mm across and about 0.75 mm along the unique direction. No destaining of the crystals was observed when the crystal was placed in dye-free mother liquor.

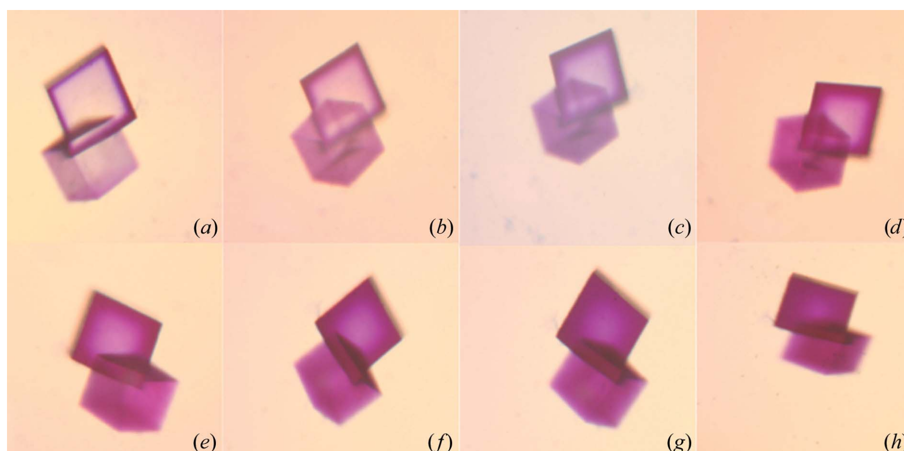

Figure 8
Concanavalin A perfused with crystal violet. A pair of concanavalin A crystals of edge dimensions about 0.60 mm are seen absorbing the dye crystal violet from their mother liquor. The total diffusion time to saturation was about 6 h.

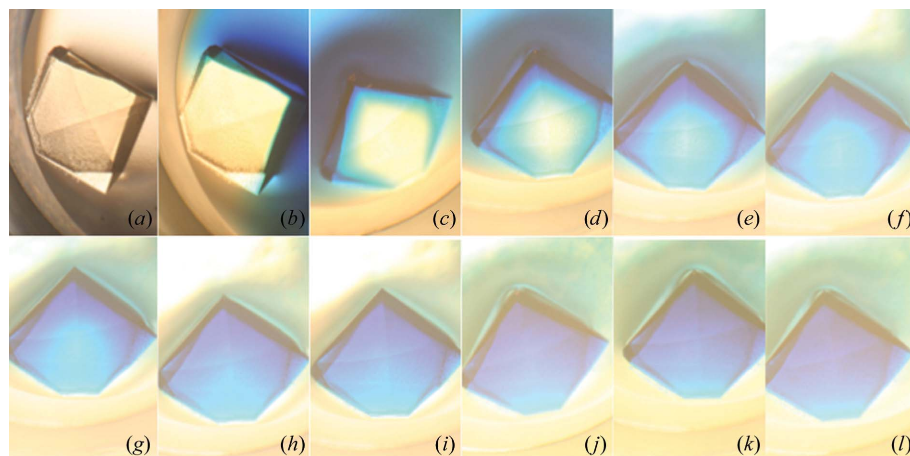

Figure 9
Thaumatin perfused with xylene cyanol. A large plate-shaped, tetragonal thaumatin crystal growing at the edge of its mother-liquor drop was exposed to xylene cyanol. The crystal is about 1.2 mm on its edges. Images (*a*) to (*l*) follow the diffusion of the dye into the crystal over a period of about 40 h.

Additional examples where strong retarding interactions occurred are presented in Supplementary Figs. S1–S5. For these combinations of protein crystal and dye, the rates of progress of the dye through the crystal ranged from about 15 µm h^{-1} to about 30 µm h^{-1}, with an average of 25 µm h^{-1}. Representative data for the experiments illustrated by Figs. 2–10 and Supplementary Figs. S1–S5 are presented in Table 1. The average penetration rate for crystals showing no significant affinity for the dye was about 75 µm h^{-1} and that for crystals for which there was significant affinity was about 25 µm h^{-1}, a threefold difference.

4. Discussion

The rates of penetration of molecules lying in the molecular-weight range 250–1000 Da into protein crystals, typified by these dyes, is a complicated matter even if strong associative interactions between the small molecules and the proteins are absent. If significant association occurs then the situation is more complex. Penetration, however, is ultimately tractable quantitatively and may even be amenable to mathematical modeling. Many of the variables involved are either experimental variables, which are therefore known quantities, or calculable entities. The size and shape of a dye, its envelope or van der Waals volume and its hydrodynamic radius would be important as well as through how small an aperture it can pass. This is complicated by the potential for multiple conformations of the dye, but most of these features can be derived from the known molecular structure of the dye. The electrostatic and hydrophobic surfaces of the dye that is exposed, which are dependent on the pH and solvent, should also be available from the structure and chemistry of the dye. The strengths of positive and negative interactions between the dye and protein surfaces as the dye moves through the unit cell might be determined experimentally for the dye and protein in solution, but this poses perhaps the most difficult challenge.

The major feature of the system that would have to be described is the shape or volume of the network of channels and spaces that permeate the crystal, and the electrostatic and hydrophobic surfaces of the protein that are exposed within the spaces. This will be entirely dependent on the protein, the crystal and the pH. If the crystal structure is known (as it is for all of the protein crystals studied here) then these features may be calculable and subject to modeling. The problem is

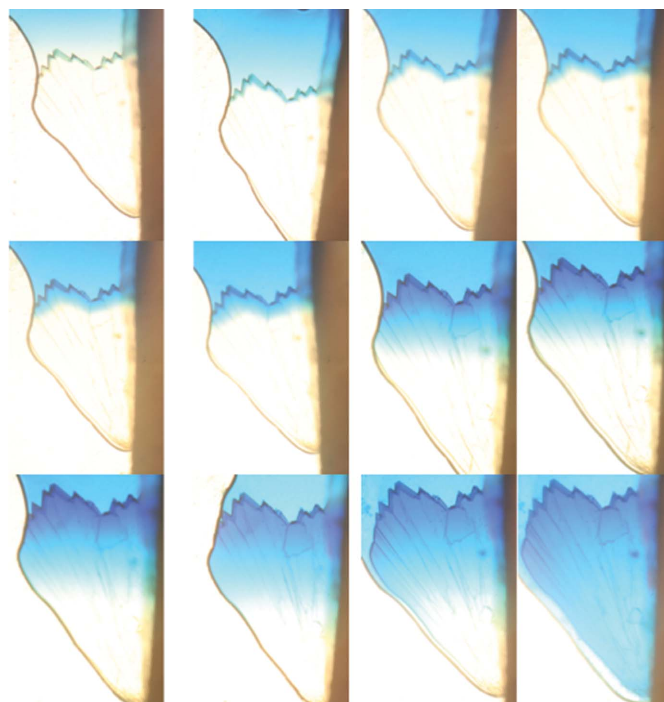

Figure 10
A thaumatin crystal grown between glass plates perfused with xylene cyanol. A thaumatin crystal, which is more or less cone-shaped by the liquid channel in which it formed, is sandwiched between two glass slides separated by 200 µm. The dye xylene cyanol was added at the surface of the large end and can be seen diffusing through the crystal towards the tip. The length of the cone is 3 mm and the total time required for the blue dye to reach the tip was about 192 h.

Table 1
Penetration rates.

Protein/dye combination	Crystal maximum dimension (mm)	Saturation time (h)	Penetration rate (μm h^{-1})	Protein/dye interaction (yes/no)
Canavalin/methylene blue	1.5	18	83	No
Canavalin/bromophenol blue	1.5	20	75	No
Canavalin/xylene cyanol	1.5	19	80	No
Canavalin/erythrosine B	1.2	22	55	No
Concanavalin A/crystal violet	0.60	6	100	No
Lysozyme/methyl green	1.0	19	53	No
Glucose isomerase/basic fuchsin	0.85	42	20	Yes
Thaumatin/basic fuchsin	0.75	47	16	Yes
Thaumatin/toluidine blue	0.70	22	32	Yes
Thaumatin/xylene cyanol (glass plates)	3.0	192	16	Yes
Thaumatin/xylene cyanol	1.2	40	30	Yes
Lysozyme/bromophenol green	0.80	36	22	Yes
Lysozyme/toluidine blue	1.2	32	38	Yes
Lysozyme/thioflavin T	6.0	Incomplete	<4	Yes

simplified somewhat by the periodicity of the network throughout the crystal.

Penetration rates will be dependent on the concentration of the dye, ion or small molecule in the mother liquor, but again these would be experimental variables. The direction of penetration through the crystal may be dependent on the symmetry of the crystal, but from our experiments this is not an important factor as penetration appears to exhibit similar rates from every face. It might, however, be important for specific crystals, such as those of extended prismatic habit (for example concanavalin B) or thin plates (for example RNase S). A final lesson of potential importance to crystallographic investigations is that in experiments involving the diffusion of small molecules into protein crystals the exposure time should probably be measured in days rather than hours.

5. Related literature

The following references are cited in the Supporting Information for this article: Koszelak *et al.* (1989), Morrison *et al.* (1984) and Smith *et al.* (1982).

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