Improved protein-crystal identification by using 2,2,2-trichloroethanol as a fluorescence enhancer

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The identification of initial lead conditions for successful protein crystallization is crucial for structural studies using X-ray crystallography. In order to reduce the number of false-negative conditions, an emerging number of fluorescence-based methods have been developed which allow more efficient identification of protein crystals and help to distinguish them from salt crystals. Detection of the native tryptophan fluorescence of protein crystals is one of the most widely used methods. However, this method can fail owing to the properties of the crystallized protein or the chemical composition of the crystallization trials. Here, a simple, fast and cost-efficient method employing 2,2,2-trichloroethanol (TCE) has been developed. It can be performed with a standard UV-light microscope and can be applied to cases in which detection of native tryptophan fluorescence fails. In four test cases this method had no effect on the diffraction properties of the crystals and no structural changes were observed. Further evidence is provided that TCE can be added to crystallization trials during their preparation, making this method compatible with high-throughput approaches.

1. Introduction

One of the essential steps in macromolecular X-ray crystallography is the identification of initial lead conditions in which the studied macromolecule crystallizes. For this, the experimenter must be able to identify crystalline structures independent of their size or the environment originating from the properties of the crystallization experiment. Furthermore, once a crystal has been detected, a reliable differentiation between salt and macromolecular crystals has to be made. A misinterpretation in one of these two steps could not only lower the chance of a successful outcome of macromolecular crystallization screening but also waste limited resources.

Analyzing the diffraction properties of crystals, allowing them to be distinguished from salt crystals, for example, is not always possible as they might be too small for mounting. In addition, often no permanent access to a suitable diffractometer is available. Therefore, several methods have been established for the differentiation of protein and salt crystals. Apart from destroying crystals by crushing, dehydration or burning, more gentle methods have also been developed, for example staining protein crystals using dyes, such as methylene blue, which noncovalently bind to proteins (Wilkosz *et al.*, 1995; Raghunathan *et al.*, 2010). More recent methods are based on fluorescence. In particular, the native fluorescence of

amino acids, especially tryptophan, is used. Alternatively, protein crystals are specifically stained with fluorophores, which are either soaked into the crystals, for example 1-anilinonaphthalene-8-sulfonic acid, or which are cross-linked to a small proportion of the protein prior to crystallization (Groves *et al.*, 2007; Judge *et al.*, 2005; Pusey *et al.*, 2015). The main advantage of these fluorescence-based techniques is that protein crystals can be detected very specifically. Protein crystals typically fluoresce brightly when illuminated by the relevant excitation wavelengths, whereas salt crystals remain dark. In addition, owing to the denser protein packing in crystals compared with precipitate, protein crystals can be identified even if they are buried within precipitate and thus are barely visible under bright light conditions (Meyer *et al.*, 2015). This enables the detection of protein crystals in a fully automated high-throughput setup (Forsythe *et al.*, 2006). With the development and commercial availability of suitable instruments, protein-crystal identification by detecting the tryptophan fluorescence of crystals is one of the most frequently used methods for the characterization of crystals in macromolecular crystallography (Dierks *et al.*, 2010; Madden *et al.*, 2011). However, it is not unusual for this method to fail because its sensitivity depends on several conditions, including the size of the crystals and the frequency and chemical environment of the tryptophan residues in the crystallized protein, as well as on the chemical composition of the crystallization trials (Desbois *et al.*, 2013; Gill, 2010).

In order to have a method in hand in case native tryptophan fluorescence fails, we searched for an alternative that is efficient, fast and affordable and can be used with the same instrumental setup as is used for the identification of protein crystals by tryptophan fluorescence. We came up with the idea of modulating tryptophan fluorescence by chemically modifying the indole side chain using 2,2,2-trichloroethanol (TCE). TCE is a small halogenic molecule which is used in Coomassie-free protein-staining techniques to detect proteins in polyacrylamide gels (Ladner *et al.*, 2004). Upon excitation with UV, TCE reacts with the indole moieties, shifting their fluorescence emission from ~ 350 nm towards visible light (~ 512 nm) (Edwards *et al.*, 2002). After the treatment of crystals of several test systems with TCE, we analyzed their fluorescence upon UV exposure and their diffraction properties and investigated their crystal structures.

2. Materials and methods

2.1. Instrumentation and material

Imaging of crystals in bright-field or under fluorescence conditions was carried out using a Nikon D5000 mounted on a Leica M205C microscope modified with an XtalLight 100 system (XtalConcepts GmbH, Germany). The XtalLight system was equipped with a mercury-vapour lamp operated at 120 W. Light from this lamp was filtered through a short-pass filter (cutoff at 320 nm) and used as a UV-light source. 96-well sitting-drop iQ crystallization plates (TTP Labtech, United Kingdom), MRC Maxi 48-well crystallization plates, Cryschem

plates (Hampton Research, USA) and CombiClover plates (Jena Bioscience, Germany) were used as crystallization plates. 2,2,2-Trichloroethanol (T54801) was purchased from Sigma-Aldrich, Germany.

2.2. Protein expression and purification

PPEP-1, CK2 α^{1-335} and HSP47 were expressed and purified as described previously (Pichlo *et al.*, 2016; Ermakova *et al.*, 2003; Oecal *et al.*, 2016). All other proteins were also recombinantly expressed in *Escherichia coli* cells and subsequently purified by affinity chromatography and size-exclusion chromatography.

2.3. Crystallization details

PPEP-1(WT) and PPEP-1(W103F) were crystallized as described previously (Pichlo *et al.*, 2016). The precipitant solution consisted of 0.1 M Tris pH 8.5, 2.0 M ammonium phosphate. CK2 α^{1-335} was crystallized by the sitting-drop vapour-diffusion method at 20°C. 48 μ l of a solution of 7.5 mg ml $^{-1}$ CK2 α^{1-335} in 500 mM NaCl, 25 mM Tris pH 8.5 was mixed with 2 μ l of a solution of 20 mM ATP-competitive inhibitor in 100% DMSO and incubated for 2 d at 20°C. Crystallization was performed by mixing reservoir solution (4.6 M NaCl, 0.1 M citric acid pH 5.5) and CK2 α^{1-335} -inhibitor mixture in a 1:1 ratio. The total drop volume was 2 μ l. Hsp47 was crystallized as described previously in 96-well sitting-drop iQ crystallization plates (Widmer *et al.*, 2012). The precipitant solution consisted of 0.1 M HEPES pH 7.5, 18% (w/v) PEG 3350, 5% (v/v) Tacsimate pH 7.0 (Hampton Research). The protein solution consisted of 9.2 mg ml $^{-1}$ Hsp47 mixed in a 1:2 molar ratio with trimeric Ac-PPGPPGPRGPPGPPG-NH $_2$ (Peptide2.0, USA). Protein_5 was crystallized in sitting drops in CombiClover plates in the presence of 65% Tacsimate pH 6.75 (16.25% Tacsimate pH 6.0, 48.75% Tacsimate pH 7.0) with 0.1 M Bis-Tris propane pH 6.75 and a drop ratio of 2 μ l 22 mg ml $^{-1}$ protein solution and 1 μ l reservoir. Protein_6 was crystallized by the sitting-drop method at 4°C. 5 mg ml $^{-1}$ protein solution was mixed with precipitant solution (1.6 M ammonium sulfate, 0.1 M MES pH 5.5, 0.01 M CoCl $_2$) in a 1:1 ratio. The total drop volume was 3 μ l. Crystals appeared after 3–4 d. Salt crystals (as confirmed by a cracking test) were obtained in conditions where the crystallization trials contained either magnesium and phosphate or zinc and sulfate. The Protein_7 crystallization trials were used to assess the fluorescence of the precipitate after TCE treatment owing to the high number of tryptophans present in this protein. A crystallization drop with a precipitant solution consisting of 0.1 M Bis-Tris propane pH 7.25, 0.9 M magnesium sulfate, 15 mM yttrium chloride is depicted in Fig. 1. Thermolysin from *Geobacillus stearothermophilus* (catalogue No. T7902, Sigma-Aldrich) was solubilized with 0.05 M NaOH to a concentration of 25 mg ml $^{-1}$ for crystallization. Thermolysin crystallization trials were set up with a total volume of 300 nl using the commercially available sparse-matrix screens Index HT, PEG/Ion HT, Crystal HT (Hampton Research) and JBScreen JCSG++ (Jena Bioscience). The precipitant solutions of the

Figure 1

Microscope images of representative crystallization trials where the identification of protein crystals using native tryptophan fluorescence failed but was successful with TCE treatment. Each row represents one crystallization trial before and after TCE treatment. The proteins are indicated on the far left. First two columns: images of crystallization trials with bright light (first column) and with UV light (second column) before TCE treatment. Third and fourth columns: images of crystallization trials with bright light (third column) and with UV light (fourth column) after TCE treatment. Further information about the conditions of certain crystallization trials is given in §2. The tryptophan frequencies of the proteins are as follows: PPEP-1(WT), 1%; PPEP-1(W103F), 0.5%; CK2α¹⁻³³⁵, 1.5%; HSP47, 1.2%; Protein_5, 0.2%; Protein_6, 2.4%; Protein_7, 2.7%

screens were either supplemented with 4 μ l H₂O (TCE-free control) or with 30 mM TCE (4 μ l of 0.5 M TCE in H₂O). Crystallization trials were incubated at 4°C. Crystals for diffraction analysis grew in conditions consisting of 40 mM KH₂PO₄, 16%(w/v) PEG 8000, 20%(v/v) glycerol or 50 mM Bis-Tris pH 6.5, 50 mM ammonium sulfate, 30%(w/v) pentaerythritolethoxylate (15/4 EO/OH) as the precipitant solution, respectively. The crystals were illuminated with UV for at least 10 min before being mounted on nylon loops. Lysozyme from chicken egg white (catalogue No. L6876, Sigma–Aldrich) was solubilized to a concentration of 30 mg ml^{−1} in 20 mM Tris pH 7.5, 200 mM NaCl. For crystallization trials with lysozyme the same procedure was used as for thermolysin but the PEG/Ion screen was replaced by the SaltRx HT screen (Hampton Research) and the plates were incubated at room temperature. Crystals for diffraction analysis grew in conditions consisting of 0.2 M Li₂SO₄, 0.1 M phosphate–citrate pH 4.2, 20%(w/v) PEG 1000 or 1.0 M LiCl, 0.1 M sodium citrate pH 4.0, 20%(w/v) PEG 6000 as the precipitant solution, respectively. Ethylene glycol was used as a cryoprotectant agent during cooling.

2.4. TCE treatment

For TCE staining of protein crystals, 100 mM TCE was dissolved in the precipitant solution used for crystallization. This solution was then added to the drops in a ratio of 1:3, resulting in a concentration of 25 mM TCE in the crystal drop. Tryptophan TCE labelling was induced by UV-light illumination of the drops for 5 min using the XtalLight 100 system.

2.5. Data collection and structure determination

Diffraction data were collected on beamlines ID29 and ID30B at the European Synchrotron Radiation Facility (ESRF), Grenoble, France or on beamline X06DA at the Swiss Light Source, Paul Scherrer Institute, Villigen, Switzerland and were processed using *XDS* (Kabsch, 2010). The structures were solved by molecular replacement with a crystal structure of PPEP-1 (PDB entry 5a0p; Schacherl *et al.*, 2015), thermolysin (PDB entry 1tlx; English *et al.*, 1999) or lysozyme (PDB entry 4b0d; Cipriani *et al.*, 2012) as the search model. Between iterative cycles of refinement using *phenix.refine* (Afonine *et al.*, 2012), missing loops as well as TCE molecules were manually built with *Coot* (Emsley *et al.*, 2010). Restraints for the TCE molecule were calculated using *phenix.elbow* (Moriarty *et al.*, 2009).

3. Results

In several macromolecular crystallization experiments we found that protein-crystal identification by native tryptophan fluorescence failed. Although most crystals turned out to be protein crystals, as shown by subsequent X-ray diffraction analysis, no tryptophan fluorescence could be detected at the outset. We therefore wondered whether we could increase the sensitivity of tryptophan fluorescence by chemically modifying the tryptophan residues of the crystallized proteins using TCE,

Table 1

Frequency of tryptophan residues in the proteins used in this study.

Protein	No. of Trp residues	No. of amino acids	Percentage of Trp residues	Molecular weight (kDa)
PPEP-1(WT)	2	198	1	21.9
PPEP-1(W103F)	1	198	0.5	21.9
CK2 α^{1-335}	5	335	1.5	40
HSP47	5	400	1.2	44.5
Protein_5	1	464	0.2	51.3
Protein_6	7	297	2.4	34
Protein_7	7	251	2.7	27.9

leading to increased fluorescence in the range of visible light, and thus enhance detection sensitivity. In order to test this, we chose crystallization trials from six different proteins where crystals were present in the crystallization drops and where tryptophan fluorescence of the crystals was only very barely detectable or completely absent (the first two rows in Fig. 1). In these cases, the very weak or even absent fluorescence was probably owing to a low content of tryptophan residues (0.2% tryptophan residues in Protein_5; Fig. 1, Table 1) in the crystallized protein, the small size of the crystals (HSP47), a combination of both [0.5% tryptophan residues in PPEP-1 (W103F)] or an unknown reason (PPEP-1). Conditions with no detectable tryptophan fluorescence were also present in crystallization experiments that contained compounds that are known to interfere with tryptophan fluorescence (Desbois *et al.*, 2013) such as metal ions (Protein_6; Co²⁺) or an organic proprietary compound absorbing light at around 350 nm (CK2 α^{1-335}).

We added TCE to each crystallization drop to a final concentration of 25 mM and directly illuminated the drop using a UV light-source system (XtalLight 100, XtalConcepts GmbH) in order to induce the reaction between tryptophan side chains and TCE. Within the first minute of UV exposure we could observe that the fluorescence of all tested crystals increased. After a 5 min exposure, all crystals brightly fluoresced under UV-light illumination. Further tests with salt crystals (Fig. 1) and with precipitate revealed that TCE treatment selectively increased the fluorescence of only protein crystals in a significant manner. Salt crystals remained dark after TCE treatment, and the precipitate showed only a minor increase in fluorescence even when the precipitated protein had a high tryptophan content (2.7% tryptophans in Protein_7; Fig. 1, Table 1). Based on these results, we concluded that protein crystals can be chemically modified with TCE.

Notably, we realized that TCE staining in the presence of collisional quenchers such as Co²⁺ was not as efficient as in the absence of these quenchers [compare PPEP-1(WT) with Protein_6 in Fig. 1]. We consistently observed a moderate reduction in fluorescence when we added 0.5 M KI to TCE-treated PPEP-1(WT) crystals. This suggests that mainly (but not only) solvent-accessible tryptophan residues are chemically modified (Ladner *et al.*, 2007). Nevertheless, TCE labelling of crystals still usefully increases the fluorescence of protein crystals (Protein_6), allowing their detection even when native tryptophan fluorescence fails.

Figure 2

TCE treatment has no impact on the structure of PPEP-1. (a) Ribbon representation of a superimposition of the asymmetric units of PPEP-1 crystals. The structure derived from native PPEP-1 crystals is shown in grey, while the structure from TCE-treated crystals is shown in blue. Tryptophan residues are shown as sticks and noncovalently bound TCE molecules next to the N-termini of PPEP-1 are depicted in orange. (b, c) A close-up view of Trp103 and Trp110 from chains A and B, respectively. All side-chain residues within a proximity of 5 Å are also presented as sticks. (d) Electron density around tryptophans. The $2F_o - F_c$ map is coloured blue and its contour level is 1σ ($0.4391 \text{ e } \text{\AA}^{-3}$). The $F_o - F_c$ map is coloured green (positive density) and red (negative density) and is contoured at 3σ ($0.400 \text{ e } \text{\AA}^{-3}$). (e) Electron density and model of a noncovalently bound TCE molecule. The TCE molecule (orange) and the surrounding amino acids from PPEP-1 (blue) are shown as sticks. TCE mainly interacts with PPEP-1 via Cl-H interactions. The $2F_o - F_c$ map is coloured blue and its contour level is at 1 r.m.s.d. The $F_o - F_c$ difference map is coloured green (positive density) and red (negative density) and is contoured at 3σ .

Table 2

Data-collection and refinement statistics for PPEP-1 crystals.

	TCE-PPEP-1	Native PPEP-1 (Schacherl <i>et al.</i> , 2015)
Data collection		
Space group	$P2_12_12_1$	$P2_12_12_1$
a, b, c (Å)	43.09, 71.67, 118.07	43.17, 71.77, 117.80
α, β, γ (°)	90, 90, 90	90, 90, 90
Wavelength (Å)	1.0	1.0
Resolution (Å)	45.6–1.38 (1.43–1.38)	45.5–1.40 (1.49–1.40)
No. of observations	492962 (48750)	310074 (45851)
No. of unique reflections	76148 (7497)	71874 (11172)
Multiplicity	6.5 (6.5)	4.3 (4.1)
Completeness (%)	100 (100)	98.2 (95.6)
R_{merge} (%)	6.4 (50.5)	5.2 (52.6)
R_{meas} (%)	6.9 (55.0)	5.9 (60.2)
$\langle I/\sigma(I) \rangle$	14.69 (2.51)	15.66 (2.48)
$CC_{1/2}$ (%)	99.9 (87.7)	99.9 (85.5)
Refinement		
$R_{\text{work}}/R_{\text{free}}$ (%)	16.3/18.4	15.6/18.2
Root-mean-square deviations (Engh & Huber, 1991)		
Bond lengths (Å)	0.008	0.006
Bond angles (°)	0.95	1.022
Average B factor (Å ²)		
All protein atoms	19.38	16.12
Water molecules	29.18	29.74
Other atoms	22.70	10.12
Ramachandran plot (%)		
Most favoured	99	98.72
Additionally allowed	1	1.28
Disallowed	0	0
Disulfide bonds	0	0
PDB code	5n12	5a0p

While we observed no macroscopic damage to crystals treated with TCE (Fig. 1), we nevertheless wanted to assess the impact of TCE treatment on the diffraction properties of the crystals. To do this, we decided to analyze the X-ray diffraction of PPEP-1 crystals, owing to their consistent diffraction properties in previous experiments (Schacherl *et*

al., 2015; Pichlo *et al.*, 2016). We observed that TCE-treated PPEP-1 crystals (seven in total) diffracted to resolution limits ranging from 1.3 to 1.7 Å, with an average of 1.5 Å. This range of resolution limits is identical to the resolution limits for native PPEP-1 crystals (Schacherl *et al.*, 2015). In addition, other relevant indicators of data quality were consistent between TCE-treated and untreated crystals (Table 2). In line with this, we observed that a small crystal of CK2 α^{1-335} containing an inhibitory compound and treated with TCE diffracted to 2.59 Å resolution (Supplementary Table S1). In comparison, a TCE-untreated CK2 α^{1-335} crystal with a slightly larger size showed comparable diffraction to 2.2 Å resolution, suggesting that TCE treatment also had no or only a very minor negative impact on the diffraction quality of CK2 crystals (Supplementary Table S1). Furthermore, the TCE-treated crystals appeared to be unchanged in their brightness and shape during X-ray exposure.

TCE-treated crystals of PPEP-1 were isomorphous to those reported previously (Schacherl *et al.*, 2015). The r.m.s.d. of a backbone alignment over 392 residues was 0.16 Å (Fig. 2a). PPEP-1 contains only two tryptophans, Trp103 and Trp110, which are both located in a flexible loop named the S-loop. This loop region was highly similar in the structures of TCE-treated and untreated crystals. In the S-loop of chain *B* some minor shifts of between 0.5 and 1 Å were observed and the indole moiety of Trp103 was tilted by 4° in the TCE-treated crystal, which is in accord with the variability that we observe for different crystal structures of PPEP-1 (Fig. 2b). All of the remaining tryptophan residues exhibited exactly the same conformation and position as in the structure of untreated crystals (Figs. 2b and 2c). Additionally, no significant alterations in the conformation of amino acids within a proximity of 5 Å to the tryptophans (Figs. 2b and 2c) and no additional electron density at the indole rings were visible (Fig. 2d). This

Figure 3

The numbers of successful lysozyme and thermolysin crystallization drops (left) and conditions (right) were counted in the presence and the absence of 30 mM TCE. For each condition, three drops with varying protein:precipitant ratios (1:2, 2:1 and 1:1) were set up. A significant influence of TCE on lysozyme or thermolysin crystallization could not be observed in either the number of drops or the number of conditions.

Table 3

Data-collection and refinement statistics for thermolysin and lysozyme crystals crystallized in the presence of TCE after UV treatment.

	Thermolysin	Lysozyme
Data collection		
Space group	<i>P</i> 6 ₁ 22	<i>P</i> 4 ₃ 2 ₁ 2
<i>a</i> , <i>b</i> , <i>c</i> (Å)	92.9, 92.9, 129.7	78.5, 78.5, 37.5
α , β , γ (°)	90, 90, 120	90, 90, 90
Wavelength (Å)	1.0	1.0
Resolution (Å)	46.47–1.39 (1.44–1.39)	31.07–1.20 (1.24–1.20)
No. of observations	1288194 (122822)	196642 (17867)
No. of unique reflections	66860 (6535)	37081 (3649)
Multiplicity	19.3 (18.8)	5.3 (4.9)
Completeness (%)	99.9 (99.9)	99.8 (99.5)
<i>R</i> _{merge} (%)	11.9 (194)	4.7 (91)
<i>R</i> _{meas} (%)	12.2 (199)	5.2 (102)
<i>I</i> / σ (<i>I</i>)	16.71 (1.52)	13.77 (1.42)
<i>CC</i> _{1/2} (%)	99.9 (64.8)	99.9 (60.9)
Refinement		
<i>R</i> _{work} / <i>R</i> _{free} (%)	14.8/16.2	15.3/17.0
Root-mean-square deviations (Engh & Huber, 1991)		
Bond lengths (Å)	0.008	0.006
Bond angles (°)	0.95	0.88
Average <i>B</i> factor (Å ²)		
All protein atoms	17.13	21.88
Water molecules	28.23	30.87
Other atoms	34.28	30.87
Ramachandran plot (%)		
Most favoured	96.5	99.2
Additionally allowed	3.5	0.8
Disallowed	0	0
Disulfide bonds	0	4
PDB code	6fsm	6fsj

also holds true for all other aromatic amino acids. Only some additional electron density for three TCE molecules located near the N-termini of both chains (Figs. 2*a* and 2*e*) allowed the differentiation of TCE-treated and untreated crystals. These TCE molecules were on the surface of the proteins and were not covalently attached to tryptophan residues, and thus could not be the reason for the increased fluorescence. They were noncovalently bound to PPEP-1 mainly *via* H–Cl interactions between the Cl atoms of TCE and the aliphatic groups of side-chain residues of PPEP-1. In summary, the results clearly show that TCE treatment had no impact on the overall structure of PPEP-1.

As TCE treatment seems to have no strong negative impact on crystal quality, we next analyzed whether TCE could also be added to freshly prepared crystallization trials in order to circumvent its addition after crystals have grown. In an initial experiment we were able to confirm that 25 mM TCE did not interfere with the crystallization of PPEP-1 and, more importantly, could be used to enhance the fluorescence of the crystals. This prompted us to quantitatively assess the impact of TCE on the crystallization of lysozyme and thermolysin under a broad range of conditions. For this, we set up crystallization trials with each of the two proteins in three different drop ratios (1:1, 2:1 and 1:2) and in the presence and absence of 30 mM TCE using commercially available screens. Here, we observed that in the majority of the trials crystals appeared independent of TCE (Fig. 3). Lysozyme or thermolysin crystallized only in the presence or in the absence of TCE in only ~10% of the conditions. We assume that this is

a result of the fluctuations that are usually observed in crystallization experiments. Importantly, as lysozyme and thermolysin crystallized in the presence of TCE as often as in its absence, we conclude that TCE has no negative impact on the probability of crystallization of these two proteins. In all of the tested conditions (25 in total), UV illumination increased the fluorescence of lysozyme and thermolysin crystals. Even at the last time point of plate inspection, four weeks after setting up the trials, UV-triggered TCE staining worked with all tested crystals. This strongly indicates that TCE was fully functional until this time point and, more importantly, this experiment suggests that TCE can be used in crystallization trials as an additive without harming the crystallizing protein.

To confirm that TCE labelling has no negative impact on the structure of the protein crystals, we also analyzed the diffraction properties of TCE-labelled thermolysin and lysozyme crystals. The thermolysin crystals were hexagonal and were isomorphous to the crystals for the majority of deposited thermolysin structures. The crystals diffracted to a resolution limit of 1.4–1.6 Å, which is slightly better than the average resolution limit for deposited thermolysin structures in the same crystal form (1.7 Å; Table 3). The lysozyme crystals were tetragonal and diffracted to 1.05–1.2 Å resolution (Table 3). A similar resolution limit was only obtained for the best 10% of isomorphous crystals deposited in the PDB. TCE treatment had no effect on the structures of thermolysin and lysozyme, and we could not find any additional electron density at tryptophans or at other aromatic amino acids. Notably, the four disulfide bonds of lysozyme were also intact, thus suggesting that UV exposure was compatible with structural integrity. Collectively, the thermolysin and lysozyme data confirm that TCE treatment does not impair the diffraction quality of protein crystals and also does not have a significant effect on crystal structure.

4. Discussion and conclusion

The determination of native tryptophan fluorescence is a very useful technique in order to identify protein crystals and distinguish them from salt crystals. The TCE treatment presented here can be used when no or only very weak native tryptophan fluorescence is detected. We show here that TCE treatment very strongly and specifically increases the UV fluorescence of crystals which show no or very little native tryptophan fluorescence. Crystals treated with TCE were macroscopically undamaged, and all of the tested crystals diffracted to resolution limits that were comparable with those of untreated crystals. The structures of PPEP-1, thermolysin and lysozyme from TCE-treated crystals were identical to the native structures and no additional electron density was observed at the indole side chains of tryptophans, suggesting that only a very small proportion of tryptophan residues were chemically modified upon TCE treatment. This is in agreement with previous MS studies showing that typically less than 10% of a tryptophan population is chemically modified upon TCE treatment and that the rate of modification also corre-

lates with the surface accessibility of tryptophans (Ladner *et al.*, 2007).

Native tryptophan fluorescence strongly depends on the chemical environment of these residues and also on the chemical composition of the crystallization buffers (Gill, 2010; Desbois *et al.*, 2013). We conclude that the increased fluorescence of crystals upon TCE treatment is the result of a small proportion of tryptophan residues which were chemically modified and thus their fluorescence was efficiently enhanced. These results demonstrate that TCE treatment is an easy, effective and affordable method of detecting protein crystals by fluorescence when native tryptophan fluorescence detection fails. Diffraction and structural analysis has so far revealed that TCE treatment is a gentle method that causes no loss of diffraction quality and does not induce structural alterations, thus making post-treatment structural analysis feasible. This suggests that TCE labelling could be used to obtain a better discrimination between protein and salt crystals, and that TCE can be added after crystal growth.

Here, we were also able to show that PPEP-1, thermolysin and lysozyme could easily be crystallized in the presence of TCE. This indicates that TCE labelling can potentially also be used in a high-throughput setup and thus further improves the already powerful method of protein crystal identification *via* tryptophan UV fluorescence.

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