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Ten Good Reasons for the Use of the Tellurium-Centered Anderson–Evans Polyoxotungstate in Protein Crystallography

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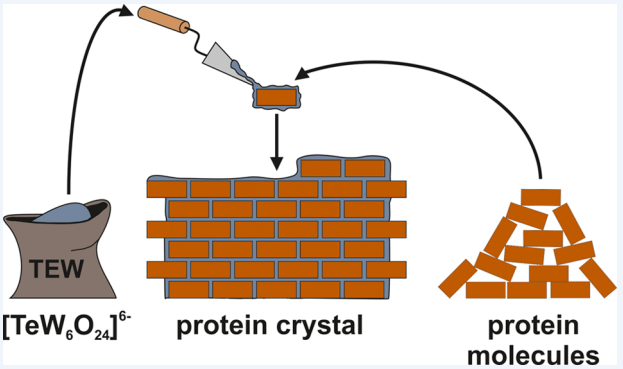
CONSPECTUS: Protein crystallography represents at present the most productive and most widely used method to obtain structural information on target proteins and protein–ligand complexes within the atomic resolution range. The knowledge obtained in this way is essential for understanding the biology, chemistry, and biochemistry of proteins and their functions but also for the development of compounds of high pharmacological and medicinal interest. Here, we address the very central problem in protein crystallography: the unpredictability of the crystallization process. Obtaining protein crystals that diffract to high resolutions represents the essential step to perform any structural study by X-ray crystallography; however, this method still depends basically on trial and error making it a very time- and resource-consuming process. The use of additives is an established process to enable or improve the crystallization of proteins in order to obtain high quality crystals. Therefore, a more universal additive addressing a wider range of proteins is desirable as it would represent a huge advance in protein crystallography and at the same time drastically impact multiple research fields. This in turn could add an overall benefit for the entire society as it profits from the faster development of novel or improved drugs and from a deeper understanding of biological, biochemical, and pharmacological phenomena.



With this aim in view, we have tested several compounds belonging to the emerging class of polyoxometalates (POMs) for their suitability as crystallization additives and revealed that the tellurium-centered Anderson–Evans polyoxotungstate $[TeW_6O_{24}]^{6-}$ (TEW) was the most suitable POM-archetype. After its first successful application as a crystallization additive, we repeatedly reported on TEW's positive effects on the crystallization behavior of proteins with a particular focus on the protein–TEW interactions. As electrostatic interactions are the main force for TEW binding to proteins, TEW with its highly negative charge addresses in principle all proteins possessing positively charged patches. Furthermore, due to its high structural and chemical diversity, TEW exhibits major advantages over some commonly used crystallization additives. Therefore, we summarized all features of TEW, which are beneficial for protein crystallization, and present ten good reasons to promote the use of TEW in protein crystallography as a powerful additive. Our results demonstrate that TEW is a compound that is, in many respects, predestined as a crystallization additive. We assume that many crystallographers and especially researchers, who are not experts in this field but willing to crystallize their structurally unknown target protein, could benefit from the use of TEW as it is able to promote both the crystallization process itself and the subsequent structure elucidation by providing valuable anomalous signals, which are helpful for the phasing step.

1. THE USAGE OF THE ANDERSON–EVANS POLYOXOTUNGSTATE AS AN ADDITIVE TO GROW PROTEIN CRYSTALS FOR X-RAY STRUCTURE DETERMINATION

1.1. X-ray Crystallography–A Powerful Method To Gain Important Structural Information

Biological macromolecules are essential for the myriad of biological functions of all living organisms. As the properties and functions of macromolecules can be derived from their 3D structure, macromolecular structure determination has gained immense importance, especially for research fields working on pharmaceutical and medicinal issues. The design and mode of action of most of the pharmaceutically active compounds depend on structural knowledge revealing relevant drug–protein interactions. This information, gained from single crystal X-ray diffraction, adds an overall benefit to the entire society as it profits from the faster development of improved drugs. According to the Protein Data Bank (PDB, www.rcsb.org) X-ray crystallography is by far the most applied method for macromolecular structure elucidation and responsible for about 90% of all PDB entries. Despite this high deposition number, crystallography is still a trial and error based method[1] and represents mainly a quite time-, cost-, and material-consuming procedure requiring typically milligram amounts of highly pure and homogeneous protein preparations. The most limiting factor is the obtaining of single crystals of sufficiently high

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quality as the crystallization process is affected by a large number of physical parameters (e.g., component concentrations, pH, temperature, ionic strength, humidity, etc.), which are partially hardly controllable, leading to the unpredictability of the crystallization outcome.

1.2. The Use of Additives To Grow Protein Crystals

One of the easiest attempts to improve the crystallization probability of a macromolecule is the application of so-called additives. Additives are small compounds or molecules that are able to interact with the protein in a crystal assembly promoting manner and thus can exhibit dramatic influence on the crystallization process. On a purely rational basis, the best additives are those that are physiologically relevant for the protein like coenzymes, substrates, inhibitors, etc. as they are able to induce more stable or favorable conformations that are in turn mostly more likely to crystallize than the ligand-free form of the protein.[2] These additives are, however, protein-specific and thus merely restrictedly applicable. Other additives like charged groups or molecules or ions are able to promote crystallization by providing intermolecular, noncovalent cross-links of electrostatic nature between protein molecules but it is mostly impossible to predict which compound under which conditions will lead to such beneficial interactions. Therefore, an universal additive with a rich repertoire of crystal packing affecting properties and addressing a larger group of macromolecules would be a groundbreaking advance in protein crystallography including all research disciplines relying on structural input.

1.3. A "Simple" Inorganic Cluster You Should Try as Crystallization Additive

During the search for a potential candidate for such a universal additive, our group examined a series of polyoxometalates[3] (POMs) with regard to their ability to enhance the crystallization rate of some proteins. POMs are polynuclear metaloxide anions with an unparalleled diversity in structure and chemistry resulting in applications in many different research areas. During our investigation, one POM archetype particularly excelled in its ability to act as crystallization additive, namely, the Anderson–Evans type polyoxotungstate (POT) $[TeW_6O_{24}]^{6-}$ (TEW). TEW led to the crystallization of two hitherto structurally unknown proteins, mushroom tyrosinase[4] from *Agaricus bisporus* (*ab*PPO4)[5−7] and aurone synthase[8−11] from *Coreopsis grandiflora* (*cg*AUS1),[12−14] and the model protein hen-egg white lysozyme (HEWL) into a previously unknown crystal form.[15] TEW was found to mediate and stabilize crystal contacts by electrostatically (including H-bonds) cross-linking protein monomers and was therefore able to facilitate crystal lattice formation. These and other properties of TEW, which are the main part of this Account, contributed greatly to protein crystallization and the subsequent structure elucidation process. Based on our success with TEW, we think that the usage of this compound as crystallization additive is highly justified presenting the only existing (but highly important) application of the pure inorganic Anderson–Evans POT on a molecular level. Therefore, this Account aims to highlight the crystallization promoting features of TEW in order to approach protein crystallographers or scientists in general who are willing to elucidate the structure of their target protein, and we address the ever increasing POM community since we describe a POM-based application. Finally, we will give an outlook about possible extension of its usage by modifying the inorganic core of TEW representing an additional favorable feature of this POM archetype.

2. THE ANDERSON–EVANS POLYOXOTUNGSTATE ARCHETYPE[16]

2.1. Inorganic Anderson-type Structure

The Anderson[17]–Evans[18] cluster is one of the pioneering POM archetypes and its structure was anticipated by J. S. Anderson in 1937; however, it was not until 1948 that the structure was crystallographically confirmed and later in 1974 finalized by H. T. Evans.[19] The Anderson–Evans polyoxoanion (Figure 1) is

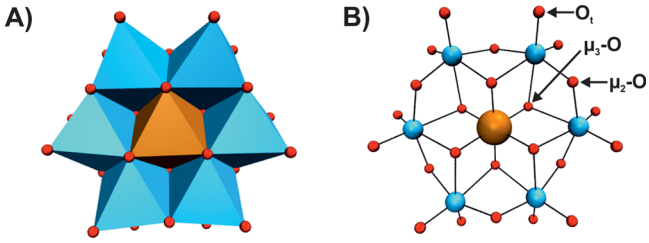

Figure 1. Polyhedral (A) and ball and stick (B) representation of $[TeW_6O_{24}]^{6-}$. Different coordination modes of the oxygen atoms are assigned in panel B. Color code: tungsten, cyan; tellurium, ochre; oxygen, red.

composed of six edge-sharing MO_6 octahedra (M = addenda atoms, Mo or W) enclosing an octahedrally arranged heteroatom XO_6 (X = most commonly transition metals) via edge-sharing leading to a planar structure that exhibits an approximate D_{3d} symmetry. Six of the altogether 24 oxygen atoms are triple-bridged (μ_3-O) connecting the heteroatom and two addenda atoms, another six oxygen atoms are double-bridged (μ_2-O) connecting two addenda atoms, and the remaining 12 oxygen atoms are terminal oxygens (O_t), which are pairwise bound to each of the six addenda atoms. There exist two types of the Anderson–Evans structure,[3] namely, the nonprotonated A-type with the heteroatom exhibiting its highest oxidation state ($[X^{n+}M_6O_{24}]^{(12-n)-}$ (e.g., X = Te^{VI}, I^{VII})) and the protonated B-type, where the heteroatom is found in a lower oxidation state and the structure contains up to six protons on the μ_3-O atoms ($[X^{n+}(OH)_6M_6O_{18}]^{(6-n)-}$ (e.g., X = Cr^{III}, Fe^{III})).

The focus of our research group lies on TEW ($[TeW_6O_{24}]^{6-}$), which was successfully applied as a crystallization additive. The POT fulfills the most important prerequisites of a crystallization additive: (i) high solubility, (ii) stability under most crystallization conditions, (iii) the ability to interact with the protein, and (iv) maintenance of the protein's integrity.[20] The application of the pure inorganic POT in protein crystallography is so far the only successful application field as this polyoxoanion is extensively employed as an inorganic building block for the synthesis of hybrid organic–inorganic POMs.

2.2. Hybrid Organic–Inorganic Anderson-type Structures

In 2002, organically functionalized Anderson-type polyoxomolybdates (POMos) gained attention when the first Anderson-type hybrid structure was reported by Hasenknopf.[21] This functionalization is achieved by replacing three or six protons of the B-type Anderson–Evans structure with organic tris-ligands (tris = tris(hydroxymethyl)methane, $(RC(CH_2OH)_3)$). Since then this research field has rapidly grown and single- or double-

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side grafted δ-, χ-, or ψ-isomers of functionalized Anderson-type POMos, $[M(OH)_6Mo_6O_{18}]^{n-}$ (M = Cu^{2+}, Zn^{2+}, Ni^{2+}, Cr^{3+}, Mn^{3+}, Al^{3+}, Fe^{3+}, Ga^{3+}), can be obtained via the rearrangement of octamolybdate or by applying a presynthesized Anderson–Evans polyanion under different reaction conditions containing the organic ligand.[16] Recently, the first tris-functionalized POT[22] has been described allowing now addition of organic ligands on the Anderson–Evans POT. Although, we have only obtained protein crystals with the solely inorganic POT, we would like to mention that also the above-mentioned hybrid structure could have beneficial effects on protein crystallization, especially when considering the synthesis of tailor-made hybrid POMs containing organic functionalities that could lead to specific interactions with the protein, for example, hydrophobic interactions (see section 4.2).

3. TEN GOOD REASONS TO USE THE ANDERSON–EVANS POLYOXOTUNGSTATE (TEW) IN PROTEIN CRYSTALLOGRAPHY

3.1. The Use of Tungsten Atoms to Solve the Phase Problem

In recent decades, POTs have been used in protein crystallography to overcome the phase problem. The introduction of heavy atoms or anomalous scatterers is the method of choice for the structure elucidation of proteins lacking a homologue structure. After the introduction of heavy or anomalously scattering atoms into the protein structure (e.g., by soaking), initial phases can be obtained by single or multiple isomorphous replacement applying heavy atoms (SIR, MIR) or by single- or multiple-wavelength anomalous dispersion using anomalous scatterers (SAD, MAD).[23−25] In all these methods, the phases are calculated based on differences in the crystals' scattering behavior, which are introduced by either the heavy atoms (SIR, MIR) or anomalous scatterers (SAD, MAD). POTs are excellent phasing tools as they represent clusters of numerous anomalously scattering heavy atoms that provide signals that significantly differ from those of the native data set enabling phase determination. Even at poor resolutions, where the single metal atom positions of metal clusters cannot be resolved or the weak signal gets lost in the noise, POT can act as a "superatom" delivering still useful phases, which is an advantage over commonly used single heavy atoms like Hg^{2+}, Au^{3+}, or $Pt^{2+/4+}$.[26,27] In the past, a series of polyoxotungstophosphates archetypes like the Wells–Dawson POT ($K_6[P_2W_{18}O_{62}]$), Keggin POT ($(H_5O_2)_3[PW_{12}O_{40}]$), and Preyssler POT ($H_{14}[NaP_5W_{30}O_{110}]$) were used to obtain heavy atom derivatives.[28−31] We successfully applied TEW as a phasing tool during the structure elucidation of mushroom tyrosinase *ab*PPO4[6,7] and HEWL.[15] A combination of molecular replacement (MR), a method deducing initial phases from the structure of a homologous protein, and SAD (MR-SAD) was applied during each structure determination. The use of the MR method alone would have been sufficient to solve those structures since good phases were derived from the respective homologue structure; however, exploiting the significant anomalous signal of TEW has improved the phase in each case and reduced model bias to a minimum, which is a common problem in MR as structural features of the homologue structure can contaminate and overlap the map of the structure of interest.

3.2. The High Solubility of TEW in Aqueous Solutions

One of the most important prerequisites of crystallization additives is, of course, a high solubility in aqueous solution as most additives are used in excess of the protein. The solubility of the sodium salt of TEW is approximately 100 mM and thus a wide range of TEW concentrations can be applied. Other POM archetypes like the Keggin and Wells–Dawson compounds are in general less soluble and thus less suitable for crystallization as they exhibit a solubility mainly in the range of 2–10 mM according to our experience. However, as the literature lacks exact description of the solubility for most POMs, the existence of POMs, exhibiting similar or even better solubility than TEW cannot be excluded (e.g., some Lindqvist-type niobates can easily reach a solubility of 20 mM). In general, the water solubility of POTs can be tuned by altering the countercation (e.g., H^+, Na^+, K^+, or Li^+).

3.3. The pH Stability of TEW

According to the PDB, proteins have been crystallized within the pH range of 2–10, whereby most of them were crystallized at pH 4–9. Thus, it is desirable that the additive largely covers this pH range in order to be widely applicable. In our experiments, TEW was stable over a period of several weeks to months at slightly alkaline and acidic pH values preserving its intact form as confirmed by crystal structures showing no hint for the formation of other protein-interacting tungsten species. The stability was tested at the common crystallization temperatures of 4 and 20 °C, respectively, and at TEW concentrations ranging from 1 to 20 mM indicating that the stability is fairly independent of the used concentration. In particular, TEW proved to be stable at pH = 7.5 (used for the crystallization of *ab*PPO4),[6,7] at pH = 5.0 (used for the crystallization of *cg*AUS1),[12,13] and at pH = 4.8 (used for the crystallization of HEWL).[15] Therefore, it can be surely recommended to use TEW from pH 4.5 to 7.5 in aqueous solution covering a relative wide pH range.

3.4. TEW Preserves the Integrity of the Protein

Crystallization additives should not harm the protein in any way that could lead to its precipitation or denaturation during the crystallization trial. X-ray structure determination and SDS-PAGE experiments of TEW-protein complexes proved so far no conformational or significant chemical changes of the respective proteins.[6,7,12−15] This should always be tested when considering the introduction of POMs into the crystallization mother liquor as some POMs tend to hydrolytically cleave proteins like lacunary POTs containing strong Lewis acids, which were shown to regioselectively cleave proteins and were therefore classified as artificial proteases.[32] The nonhydrolytic activity of TEW is given by the circumstance that the central heteroatom, tellurium, is incorporated in the planar disk-shaped Anderson structure, where it is shielded by the POM framework (Figure 1) and is thus not able to interact directly with the protein. This shielding of the central heteroatom is also verified for the Anderson–Evans type POMo analogues $FeMo_6$,[33] $MnMo_6$,[33] $GaMo_6$,[34] $CrMo_6$,[35] and the Anderson–Evans like VMo_6[36] system, which proved to be hydrolytically inert. Thus, even in the presence of stronger Lewis acids within the Anderson–Evans framework, no hydrolytic activity on the protein was observed.

It must be noted that two biomedicinal studies about TEW exist reporting that $[TeW_6O_{24}]^{6-}$ is a potent inhibitor of acetylcholineesterase (electric eel).[37] In addition, TEW was tested on two isoenzymes of alkaline phosphatases including

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tissue specific calf intestine alkaline phosphatase and tissue nonspecific alkaline phosphatase, where TEW showed activity against tissue nonspecific alkaline phosphatase. Similarly, chitosan−$[TeW_6O_{24}]^{6-}$ was proved to be a potent inhibitor of calf intestine alkaline phosphatase.[38] However, the exact binding site and interaction of TEW with the enzyme and thus the mode of inhibition remains elusive.

3.5. The Negative Net Charge of TEW Ensures Electrostatic Interaction with the Protein

The total net charge of a POM depends, inter alia, on the choice of the heteroatom as the higher its oxidation state is the lower the charge of the complex will be. In the case of TEW ($[TeW_6O_{24}]^{6-}$), the heteroatom is Te^{6+} giving rise to a total net charge of −6. This high negative charge is predestined for the interaction with positively charged patches of proteins, and indeed in all TEW containing crystal structures, TEW was found at positively charged regions. Thus, TEW should theoretically target a wide range of proteins as only positively charged protein surface regions are needed for the interaction. The TEW−protein interactions are mostly composed of electrostatic charge−charge interactions (interactions with the positively charged amino acids lysine and arginine) and H-bonds. As TEW has a relatively large size, the high negative charge is distributed over a wide area enabling TEW to electrostatically interact with large protein portions and interacting with numerous amino acid residues increasing both the probability and strength of TEW−protein interactions. This represents a clear advantage over commonly used (protein bridging) additives like small molecules or ions carrying a relatively small charge as they are only able to interact with a small and limited number of amino acid residues, which in turn leads to a reduced affinity toward the protein in comparison to TEW.

3.6. The Size and Shape of TEW Offers Different Variants of Protein−Protein Bridging

The average dimensions of the Anderson−Evans anion measures approximately 9 × 9 × 3 (Å^3) indicating the appreciable size of the anion and its planar structure. X-ray structures of the TEW−protein complexes[6,7,12−15] revealed that both the size and planar structure are very advantageous during crystallization. The relatively large size of TEW provides a certain distance between the protein molecules upon protein−protein bridging and thus prevents steric interference between the molecules, which could be a problem when using small molecules as cross-linking additives (Figure 2). This feature is even more important when two electrostatically repulsive protein patches are linked as this TEW-mediated distance could lead to reduced long-range repulsion forces and at the same time might increase short-range attraction forces, which are crucial for the nucleation process.

Due to TEW's planar structure, this distance can vary depending on TEW's orientation leading to bridged monomer−monomer distances in the range of about 6−14 Å (Figure 3). Therefore, different orientations of TEW can induce a certain versatility in protein−protein bridging, which could beneficially affect the crystal packing (by offering more freedom in cross-linking).

3.7. The Symmetry of TEW as a Beneficial Factor for Protein−Protein Bridging

It was demonstrated that trigonal ($[W_3O_2(O_2CCH_3)_6]^{2+}$ with D_3 symmetry) and pentagonal ($[NaP_5W_{30}O_{110}]^{14-}$ with D_5

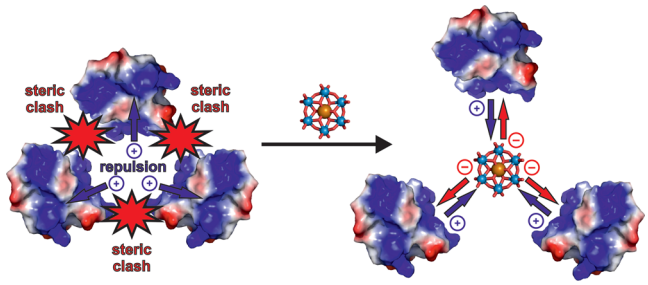

Figure 2. Schematic illustration of the "electrostatic spacer effect" of TEW. On the left of the figure, a scenario where three protein patches (depicted as electrostatic Coulombic surfaces with blue = regions of positive potential, white = neutral potential, and red = negative potential) are coming close together is illustrated. This situation can lead either to steric interference (indicated by a red 10 rays star) or, if the patches are electrostatically equal, to electrostatic repulsion (indicated by blue arrows) with both cases leading to no crystal contacts. However, in the presence of the negatively charged TEW (illustrated as ball and stick, color code tungsten, cyan; tellurium, ochre; oxygen, red), the protein patches are electrostatically cross-linked (indicated by blue and red arrows) and at the same exhibit an appropriate distance to each other preventing any steric interference.

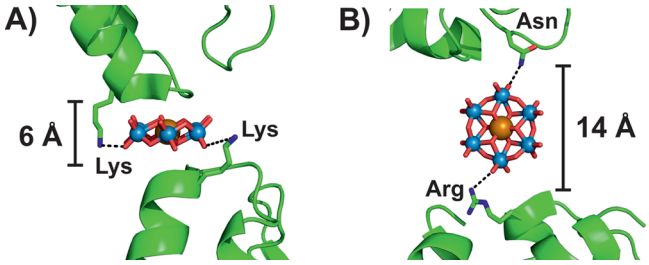

Figure 3. Protein−protein bridging by TEW in different orientations. (A) Two protein molecules (*ab*PPO4) are bridged via TEW lying vertically between them resulting in a small protein−protein distance. (B) TEW is positioned horizontally at the interface of two protein molecules (HEWL) leading to a larger distance between them. The protein molecules are shown as green cartoons, and TEW is depicted in ball and stick representation. Color code: carbon, green; nitrogen, blue; tungsten, cyan; tellurium, ochre; oxygen, red.

symmetry) POMs bind selectively at the crystallographic 3-fold and 5-fold-axis of the riboflavin synthase structure, respectively.[39] Thus, the symmetry can play a crucial role in POM-mediated protein−protein cross-linking by selectively directing the POM binding site and thus its binding behavior. However, this is only possible if the internal symmetry of the POM correlates with the protein's symmetry within the crystal. This "symmetry-effect" was also observed for TEW during the crystallization of mushroom tyrosinase *ab*PPO4 where both TEW anions in the structure were located on the same 2-fold-axis. The symmetry of TEW is approximately D_{3d} containing three C_2 axes and was thus compatible with the crystal's symmetry. By directing the binding position of TEW, the symmetry has also an impact on the degree of protein−protein cross-linking because TEW being situated on a 2-fold axis results in an environment where it is surrounded by two protein molecules, which can be bridged. Therefore, symmetry could also induce the situation where TEW is located on a crystallographic 3-fold axis leading to the cross-linking of three protein molecules. It has to be noted that during the cocrystallization of both HEWL and *cg*AUS1 with TEW, the TEW anions were not exactly or not at all located on

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crystallographic axes indicating a rather random TEW binding; however, the anions still bridged partially more than two protein molecules.

3.8. The Potential of TEW-Mediated Crystallization to Increase Crystal Quality

The ability of TEW to improve the crystal quality and thus the resolution has been observed for *cg*AUS1, which was crystallized in three crystal forms, two in the absence and one in the presence of TEW.[12] All crystal forms were obtained under almost identical crystallization conditions; however, the replacement of $MgCl_2$ by TEW as crystallization additive increased the resolution dramatically by up to 1.0 Å.[14] X-ray structure analysis revealed that the crystal contacts of *cg*AUS1−TEW are more specific than those of the TEW-less crystal forms, which results in an increase of symmetry and decrease of the number of protein molecules within the asymmetric unit (ASU). All crystal forms were built up of the same crystallographic dimer; however, in the TEW-less structures these dimers formed a tetrameric and octameric arrangement within the ASU, respectively, whereas the ASU of the *cg*AUS1−TEW structure only contained this crystallographic dimer. In the *cg*AUS1−TEW structure, two TEW anions mediate new crystal contacts with one TEW strongly stabilizing the crystallographic dimer, which seems to be the reason for the higher crystal quality as the TEW-mediated contacts, especially the dimeric contact, are by far stronger than other rather unspecific contacts within the structure. This leads to a dominating adhesion mode between the proteins (dominated by the TEW-mediated contacts) improving the diffraction behavior of the crystal.[40] This is clearly not the case in the TEW-less crystal structures, which lack a preferred adhesion mode and thus exhibit many partially unspecific protein−protein contacts leading to a decreased crystal quality. A similar observation was made in the case of *ab*PPO4. After crystallizing this enzyme in the presence of TEW,[6,7] we very recently obtained crystals without TEW but of clearly lower quality (2.76 Å vs 3.25 Å) most likely due to similar reasons as indicated above demonstrating that the TEW-mediated contacts are crucial for crystal quality in those cases.[41]

3.9. The Ability of TEW To Induce Heterogeneous Crystallization

TEW was reported by our group to mediate "heterogeneous crystal formation". Mushroom tyrosinase *ab*PPO4 was crystallized in the presence of TEW and resulted in the crystallization of both the latent (64 kDa) and active form (44 kDa) of this enzyme within one single crystal.[6] The crystal structures of both forms were unknown until then and with the use of TEW "two birds were killed with one stone". Each heterodimer (latent and active *ab*PPO4) is on the one side connected to its symmetry mate via an usual protein−protein contact and on the other side linked to the next heterodimer by a TEW-mediated contact composed of two TEW molecules (Figure 4). Two monomers of each *ab*PPO4 share one TEW molecule, which is located on a crystallographic 2-fold axis. This pattern is the structural basis for the entire crystal and demonstrates the possibility to crystallize two protein forms of clearly different size in one single crystal using TEW.

3.10. The Geometric and Functional Flexibility of TEW

In the frequently mentioned *cg*AUS1−TEW structure, one TEW molecule is unexpectedly covalently bound to the protein leading to the formation of a new TEW-derived cluster with the

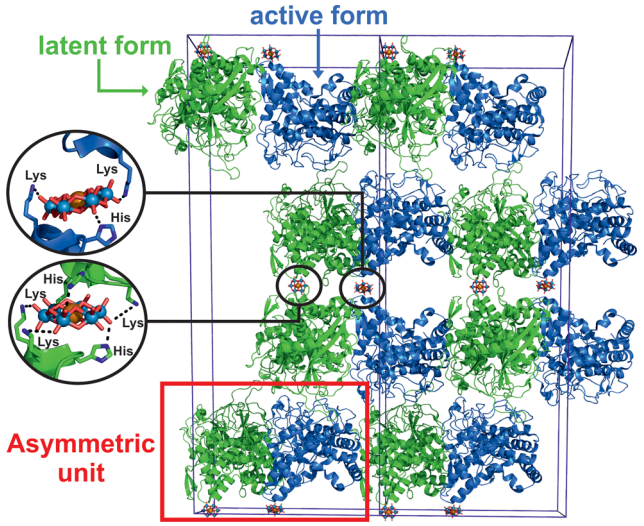

Figure 4. TEW-mediated heterogeneous crystallization of *ab*PPO4. A section of the crystal packing of *ab*PPO4−TEW is illustrated as a 1 × 2 × 1 supercell (indicated by a blue cell). Both the latent (green cartoon) and the active form (blue cartoon) of *ab*PPO4 are crystallized in one single crystal and thus present in one asymmetric unit (indicated by a red box). The TEW-mediated bridging of two heterodimers is visible, and the TEW−protein interactions are illustrated in the round insets on the left side. The protein is depicted as cartoon and TEW as ball and stick. Color code: carbon, green/blue; tungsten, cyan; tellurium, ochre; nitrogen, dark blue; oxygen, red.

formula $[TeW_6O_{24}O_2(Glu)]^{7-}$ (Figure 5), where the bond is closed between two tungsten atoms and the two carboxylic

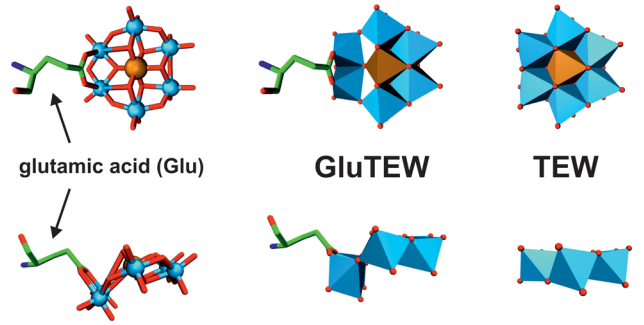

Figure 5. Covalent binding of TEW to *cg*AUS1. The carboxylic oxygen atoms of a glutamic acid (Glu157) bind covalently to two tungsten atoms of TEW accompanied by a rearrangement within the Anderson−Evans structure resulting in a bent structure named GluTEW (illustrated in the left as ball and stick and in the middle as polyhedra). For comparison, the normal Anderson−Evans structure is depicted on the right in a matching orientation as polyhedra. Color code: carbon, green; nitrogen, blue; tungsten, cyan; tellurium, ochre; oxygen, red.

oxygen atoms (O_2 in the formula) of a glutamic acid (Glu in the formula). This is so far unique as covalent bonds were only observed in experiments where the POM was *in situ* assembled in the course of the crystallization experiment and not upon the addition of the intact cluster. The covalent bond between TEW and *cg*AUS1 is accompanied by a structural rearrangement within the planar Anderson−Evans structure leading to an unprecedented bent structure (Figure 5). It was suggested that the covalent bond was sterically enforced by the environment of TEW, which is located within a highly positively charged

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cleft, by strong interactions with the surrounding amino acid residues. Therefore, it appears that in this case TEW was able to structurally adapt to the proteinogenic environment in order to fit into the binding cleft. Covalent TEW binding did not alter the overall structure of the protein as indicated by the comparison with TEW-less *cg*AUS1 structures but instead dramatically influenced its crystallization as discussed under reason 3.8. The here provided evidence of the high flexibility of TEW in both geometrical and chemical regards further encourages its use in protein crystallography. The ability to covalently bind to protein residues can lead to the stabilization of flexible protein regions, for example, loops, and thus enhance the crystallizability of proteins suffering from high structural mobility.

4. THE ANDERSON–EVANS POT CAN BE FURTHER TUNED FOR ITS APPLICATION IN PROTEIN CRYSTALLIZATION

We have demonstrated the beneficial effects of TEW as a powerful additive in protein crystallization; however, modifications of the Anderson–Evans structure are possible by (i) changing the central heteroatom resulting in a different net charge, (ii) attaching organic functionalities to the Anderson–Evans core enabling other than electrostatic interactions, and (iii) attaching hydrophobic alkyl chains for potential interaction with membrane proteins as membrane proteins represent the real bottleneck in macromolecular crystallization.

4.1. Tuning of the Net Charge by Selection of the Central Heteroatom

The possibility to tune the total negative net charge of the Anderson–Evans structure by selecting different heteroatoms exhibiting different oxidation states allows the synthesis of clusters with an even higher negative net charge as the one of TEW. The ions Mn^{4+}, Sb^{5+}, Ir^{4+}, and Pt^{4+} have been incorporated in the Anderson–Evans core as heteroatoms leading to net charges of −7 and −8.[16] In general, care should be taken when incorporating metals of lower oxidation states (e.g., Mn^{II}, Ni^{II}), as they tend to form the protonated B-type of the Anderson–Evans structure leading to a decreased net charge (−4) in comparison to TEW. An increase in total negative net charge is accompanied by a higher charge density, which could increase the affinity of this derivative toward positively charged proteins.

4.2. Hybridization with Various Organic Functionalities To Target Special Protein Sites

The ability to decorate the inorganic TEW with organic functionalities could be used to synthesize tailor-made Anderson–Evans type structures that could address specific protein sites via their attached organic entity. The Anderson–Evans type polyoxomolybdate has explicitly been decorated with a wide variety of tris-ligands (this functionality represents the basis for further modifications) through either pre- or postfunctionalization using different procedures (Figure 6).[16,42] The variation in tris-ligands includes alkyl chains of differing lengths, aromatic ligands, ligands with remote binding sites, and ligands with terminated functional groups. Very recently it became possible to tris-functionalize the Anderson–Evans POT core allowing attachment of organic functional groups.[22] For example, Anderson–Evans POTs decorated with aromatic ligands could target regions on the protein's surface with exposed aromatic residues leading to hydrophobic $\pi-\pi$ or related stacking interactions, which could support the electrostatic interactions or enable the binding of such clusters to very hydrophobic surface regions.

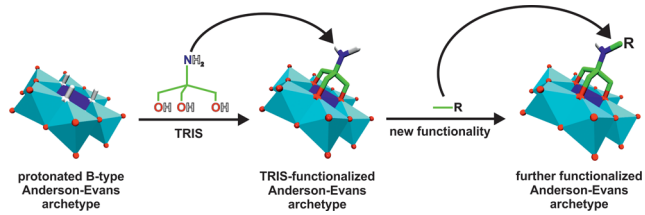

Figure 6. Schematic illustration of the synthesis of an organically functionalized Anderson–Evans structure. The TRIS-functionalization step usually takes place at the protonated B-type Anderson–Evans structure (protons are illustrated as white sticks on the left). The resulting TRIS-functionalized structure (in the middle) can then be further functionalized with another organic functionality (indicated as R) leading to a wide range of organic–inorganic hybrid structures (on the right). Color code: carbon, green; addenda atom, cyan; heteroatom and nitrogen, blue; oxygen, red; hydrogen, white.

4.3. Attachment of Large Hydrophobic Moieties on the Anderson–Evans Polyoxotungstate Core Structure Could Lead to the Solubilization of Membrane Proteins and Consequently to Their Crystallization

Furthermore, the attachment of large hydrophobic moieties like long alkyl chains on the Anderson–Evans POT could lead to a POM-based detergent applicable in membrane protein crystallization. Detergents are surface active agents capable of mediating contacts between surfaces differing in polarity, such as hydrophilic and hydrophobic surfaces. They are used in membrane protein crystallography to solubilize the membrane lipid bilayer as most membrane proteins are not soluble in aqueous solutions and thus tend to precipitate due to their hydrophobic domains. Therefore, an Anderson–Evans structure with at least one attached long alkyl chain (in addition to commonly used detergents) could be worth trying in this regard as the hybrid POM could provide valuable protein–protein cross-links (between nonlipid domains) and at the same time stabilize the membrane part of the protein via hydrophobic interactions.

5. OUTLOOK

The application of TEW in the field of protein crystallography will hopefully grow in the future providing crystal structures of proteins for which structures are unknown to this date. The recent successful applications of TEW as crystallization additive suggest that future utilization should bring benefits to several fields like pharmacology, medicine, inorganic chemistry, and especially structural biology, all of them depending on the input from 3D structures. Further systematic investigation of TEW–protein interactions will open new, perhaps today unforeseen, directions.

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Notes

The authors declare no competing financial interest.

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Annette Rompel was born in Goslar, Germany. She studied chemistry at the Westfälische Wilhelms University of Münster where she received her doctoral degree. Besides research at the University of California, Berkeley, and the Lawrence Berkeley National Laboratory, she was a visiting scientist at the RIKEN, Institute of Physical and Chemical Research, Sendai, Japan, and the University of Southern Denmark, Odense. Since 2008, she is the head of the Department of Biophysical Chemistry at the University of Vienna. Her main research interests are the structure and function elucidation applying synchrotron based techniques of metal containing enzymes from natural sources. In the last years, her strong interest in combining inorganic chemistry with biochemistry led her to work on the synthesis and characterization of polyoxometalates as powerful additives in protein crystallization.

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■ ABBREVIATIONS

*ab*PPO4, mushroom tyrosinase 4 (*Agaricus bisporus*); *cg*AUS1, aurone synthase from the yellow blossom leaves of *Coreopsis grandiflora*; GluTEW, TEW covalently bound to a glutamic acid; HEWL, hen-egg white lysozyme; PDB, Protein Data Bank; POMo, polyoxomolybdate; POMs, polyoxometalate; POT, polyoxotungstate; R-Tris, R-C(CH_2OH)$_3$; TEW, $[TeW_6O_{24}]^{6-}$

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