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Phase Determination Using Halide Ions

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Summary

A short soak of protein crystals in cryosolution containing bromides or iodides leads to incorporation of these ions into the ordered solvent shell around the protein surface. The halide ions display significant anomalous signal, bromides in the vicinity of the absorption edge at 0.92 Å, and iodides at longer wavelengths, e.g., provided by the copper sources. Bromides can, therefore, be used through multiwavelength anomalous diffraction or single-wavelength anomalous diffraction (SAD) techniques and iodides through SAD or multiple isomorphous replacement (MIRAS) phasing. The halide cryosoaking approach involves very little preparative effort and offers a rapid and simple way of solving novel protein crystal structures.

Key Words: Anomalous scattering; MAD; SAD; phasing; halides; bromides; iodides.

1. Introduction

The idea of introducing halide ions to protein crystals originated from the observation that in the crystals of lysozyme, obtained from the standard solution containing 1 *M* of NaCl, several chloride ions reside in the ordered solvent region around the protein molecule ***(1)***. Chlorine is a relatively light element and, although it has sometimes been used as a vehicle for phasing ***(2–4)***, its heavier analogs, bromine and iodine, are much better suited as anomalously scattering heavy atoms for phasing crystal structures ***(5)***. Subsequent trials confirmed that heavier halides, bromides and iodides, can also be introduced to protein crystals, if their salts are present in the crystallization medium ***(6)*** or in the cryoprotecting solution ***(7,8)*** and this approach has been proposed as one of the general methods of solving protein crystal structures ***(9)***. A number of novel protein structures have recently been solved by this approach, and some examples are listed in **Table 1**.

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Table 1
Some Novel Structures Solved With Soaked Halides

Protein	Number of residues	Resolution phas/ref (Å)	Method	Ions	Soak	Time (s)	PDB code	Reference
R11 of Man6P/IGFII receptor	286	2.2/1.75	SIRAS	16 I	1 *M* KI	60	1E6F	***(25)***
TIM, *Caenorhabditis elegans*	550	2.0/1.7	SAD	12 I	0.5 *M* NaI	600	1MO0	***(26)***
GRIP1 PDZ6-peptide	97	1.9/1.5	MAD	3 Br	1 *M* NaBr	30	1N7E	***(27)***
Drosophila NLP-core	540	2.8/1.5	MAD	8 Br	0.5 *M* NaBr	20	1NLQ	***(28)***
Noggin	345	2.4/2.4	MAD	10 Br	1 *M* NaBr	30	1M4U	***(29)***
APS kinase	844	1.9/1.43	MAD	13 Br	0.75 *M* NaBr	45	1M7G	***(30)***
KTN	294	3.1/2.85	MAD	4 Br+8 I	1 *M* NaBr/NaI	60	1LSU	***(31)***
NC1 bovine eye lens	2740	2.2/2.0	MAD	33 Br	0.5 *M* KBr	60	1M3D	***(32)***
S100A3	202	2.7/1.7	MIRAS	2 I	0.5 *M* KI	30	1KSO	***(33)***
Interleukin-22	358	1.92/1.9	SIRAS	10 I	0.125 *M* NaI	180	1M4R	***(34)***
Phenylalanine hydroxylase	297	2.4/1.74	MAD	7 Br	0.33 *M* NaBr	50	1LTU	***(35)***
NC1 human placenta	1372	2.5/1.9	MAD	25 Br	1 *M* NaBr	30	1LI1	***(36)***

Doc1/Apc10 subunit	442	2.2/2.2	SAD/SIRAS	5 Br	1 *M* LiBr	30	1GQP	***(37)***
Transcriptional repressor	142	2.2/1.5	MIRAS	2 I/3 Br	0.35 NaI/ 0.5 NaBr	n/a	1IRQ	***(38)***
Salmonella SicP-SptP	730	2.5/1.9	MAD	31 Br	2 *M* NaBr	30	1JYO	***(39)***
β-Defensin-1	72	1.5/1.2	SAD	6 Br	0.5 *M* KBr	30	1IJU	***(40)***
IGF-1	70	1.8/1.8	MAD	1 Br	1 *M* NaBr	30	1IMX	***(41)***
Peroxiredoxin 5	161	1.7/1.5	MAD	5 Br	1 *M* NaBr	30	1HD2	***(42)***
TonB	152	2.0/1.55	MAD	4 Br	1 *M* KBr	50	1IHR	***(43)***
Frizzled CRD	762	2.0/1.9	MAD	9 Br	0.5 *M* NaBr	40	1IJX	***(44)***
Thiamine pyrophosphokinase	638	2.0/1.8	MAD	12 Br	1 *M* NaBr	45	1IG0	***(45)***
PSCP	375	1.8/1.0	SAD	9 Br	1 *M* NaBr	30	1GA6	***(46)***
IPP5C	347	2.0/2.0	SIRAS	5 I	0.15 *M* KI	n/a	1I9Y	***(47)***
Acyl protein pyrophosphokinase	464	1.8/1.5	SAD	22 Br	1 *M* NaBr	20	1FJ2	***(48)***
β-Defensin-2	164	2.0/1.35	MAD	9 Br/9I	0.25 *M* KBr/KI	60	1FD3	***(49)***
GAF domain YKG9	224	2.8/1.9	MAD	7 Br	0.5 *M* NaBr	45	1F9M	***(50)***
NEIL1	364	2.3/2.1	MIRAS	8 I	0.25 *M* NaI	300	1TDH	***(51)***

Only novel structures solved with soaked halides are included in the table. Both Br MAD/SAD and I SAD/SIRAS can be equally successful, in spite of the fact that the Br soaks seem to be more popular.

Both bromine and iodine are known as useful elements for heavy-atom derivatization of macromolecules. In particular, bromouracil is almost isostructural with thymine, and has often been used for multiwavelength anomalous diffraction (MAD) phasing of crystal structures of nucleotides ***(5)***, in analogy to the role of selenomethionine in MAD phasing of protein crystals (*see* Chapter 5 of volume 1). Bromine has one electron more than selenium, and its anomalous scattering properties are similar with an X-ray absorption edge at 0.92 Å (selenium at 0.98 Å), easily achievable at most MAD-capable synchrotron beam lines. Iodine has for a long time been used as a classical heavy atom for multiple isomorphous replacement (MIR) experiments, e.g., after iodination of tyrosines with *N*-iodosuccinimide ***(10)***. Its X-ray absorption edges are not easily accessible, but iodine with its 53 electrons displays the anomalous scattering effect of about seven electron units at the Cu Kα wavelength of 1.54 Å.

2. Materials

The only material necessary after crystals of the native protein have been obtained is the halide (bromide or iodide) salt of alkali metal, lithium, sodium, or potassium. A variation of the halide-soaking approach has been proposed ***(11,12)*** where cesium or rubidium halides were used, providing an additional anomalous signal from these two cations, also bound at the protein surface (*see* **Note 1**). Another proposed variation involves triiodides ***(12,13)***, requiring elemental iodine dissolved in KI.

3. Methods

3.1. Soaking Procedure

The soaking procedure is very simple and consists of submerging the native crystal for a short time in the mother liquor containing simultaneously both a cryoprotectant and an appropriate halide salt, before freezing it for diffraction data collection.

The halide ions diffuse into protein crystals very quickly. Experiments with variable soaking times have shown that soaking times as short as 10–15 s are sufficient. Longer soaking times may degrade the crystal diffraction power, but sometimes led to crystalline phase transition (*see* **Note 2**), or actually extended the resolution limit of diffraction ***(14)***.

The concentration of salt in the soaking solution plays a more important role than a prolonged soaking time. A high halide concentration increases the number of sites and their occupancy. However, not all crystals tolerate high concentrations (up to 1 *M*) of halide salts and their crystalline order may rapidly deteriorate. The optimal concentration can only be evaluated empirically. If the crystals visibly crack or shatter when observed under the microscope, or if the initial diffraction image shows signs of significant deterioration, the concentra-

tion of halide salt in the cryosolution should be diminished. The first trials can be performed with 1 *M* concentration, but successful phasing has been obtained with salts diluted to 0.2 *M*. The triiodide soaks require much more diluted solutions, in the order of 10–20 m*M* of KI_3 ***(12)***.

It may be advantageous to preserve the content and concentration of the initial mother liquor and add the measured amount of the halide in the form of a solid salt. If the crystallization liquor contains a high concentration of another salt, such as ammonium sulfate, it may be beneficial to substitute part of that salt with a halide, preserving the overall ionic strength of the solution and decreasing the adverse competitive effect of other anions.

Some salts at very high concentration may serve as cryoprotectants ***(15)*** and it may be worth checking whether the crystal can be successfully frozen if the concentration of halide salt is increased to greater than 2 *M*.

3.2. Phasing

In general, the structure solution of diffraction data from halide-soaked crystals may proceed along well-established protocols for utilization of the anomalous signal in MAD, single-wavelength anomalous diffraction (SAD), or MIRAS techniques, and any of the suitable programs can be used. However, in contrast to the popular MAD approach based on selenomethionines, the number of identifiable anomalous scatterer sites cannot be predicted. The halide-soaked crystals always have a large number of sites with variable occupancies, but only those sites with significant occupancy are useful for the evaluation of protein phases. In general, the number of useful sites is roughly proportional to the surface area of the protein molecule, but other factors (*see* **Note 3**) obviously play important roles.

The anomalous signal of bromides can only be utilized if diffraction data are collected at the synchrotron beam line, where the X-ray wavelength can be adjusted to the vicinity of the Kα absorption edge of bromine (0.92 Å). The fluorescence spectrum recorded from the crystal soaked in the bromide salt will always show the Br Kα absorption edge, resulting from the excess of bromides in the cryosolution, regardless of the number of bound bromide sites. The presence of bound bromides (or iodides) can be confirmed from the clear anomalous signal present in the diffraction data. The anomalous signal of iodine is more pronounced at longer wavelengths; its f″ contribution at the Cu Kα wavelength is about seven electron units, so that it can be used with data collected at the laboratory X-ray sources, not only at synchrotron facilities.

3.2.1. Location of Anomalous Scatterers Sites

As usual in all methods based on heavy or anomalously scattering atoms, the first step in crystal structure solution is the location of these atoms, i.e., halide

ions in the approach discussed here. This can be done with either Patterson or direct methods, utilizing the anomalous or isomorphous (dispersive) differences, as appropriate (*see also* Chapters 10–12). If only single-wavelength data are available, the anomalous (Bijvoet) differences have to be used. If in addition a native dataset exists, the classic SIRAS (single isomorphous replacement with anomalous scattering) method can be applied, and the halide sites can be identified from the anomalous or isomorphous differences. If MAD data are collected on bromide-soaked crystal, various combinations of anomalous and dispersive differences can be utilized, as well as the properly estimated diffraction contributions of anomalous atoms, F_A.

Because the number of expected halide sites cannot be predicted, they can be selected, e.g., by comparing peaks in several putative direct methods solutions. Equivalent sites (*see* **Note 4**) present in several independent direct methods trials can be accepted with confidence. Usually after the first round of phasing it is possible to identify more sites from the appropriate (anomalous or isomorphous) difference Fourier synthesis. In practice, weaker sites can be iteratively added as long as they increase the phasing power and enhance the interpretability of the resulting electron density maps.

3.2.2. Evaluation of Protein Phases

After a number of halide sites are identified, it is possible to evaluate the protein phases, perform phase improvement (solvent flattening and related density modification procedures), and calculate the initial electron density map. This step can be performed separately, or can be a part of the integrated software system, such as SOLVE ***(16)***, autoSHARP ***(17)***, CNS ***(18)***, SHELXD/E ***(19,20)***, or BnP ***(21)***. At the stage of protein phase evaluation, in particular after density modification, there should be a contrast in the phasing statistics, particularly in the map interpretability, between the two enantiomorphs.

Obviously, if the first automatic attempt is successful, and leads to an interpretable electron density map, there is no need for any further proceedings. If the initial map is not satisfactory for either enantiomer, the phasing procedure can be modified and repeated. The possible changes involve inclusion of more heavy atom sites, a different resolution limit, or changing the program used.

If the significance of measured anomalous differences does not extend to the full-resolution limit of diffraction data, it may be beneficial to perform the phasing procedure at a lower resolution limit, and subsequently extend the phased data at the density modification step ***(22)***. Various programs use different algorithms, some employ simpler but quicker approaches, some are more elaborate but slower. It is usual to start from a quick approach, and switch to more sophisticated program if the other attempts failed. However, the individual crystallographer's experience with a particular program is often important in obtaining final success.

4. Notes

1. In addition to the use of bromides and iodides, it has been proposed that heavier alkali metal cations, Rb ***(11)*** and Cs ***(8,12)***, soaked into protein crystals can also be used for phasing. The procedure is analogous to the use of halides. The anomalous scattering properties of Rb are similar to those of Br (with the X-ray absorption edge at 0.87 Å) suitable for MAD phasing. The properties of Cs are analogous to I, with a substantial anomalous signal at a wavelength of 1.54 Å and longer.
2 It has been observed that soaking native crystals in concentrated solutions of salts sometimes causes crystal lattice transitions. The orthorhombic $P2_12_12_1$ crystals of PSCP ***(23)*** underwent transformation to the hexagonal space group $P6_122$ after short soaking in 1 *M* solution of NaBr. The crystals of human peroxiredoxin 5 ***(24)*** in a twinned monoclinic form after 30 s soak in 1 *M* NaBr changed to the tetragonal form, diffracting to higher resolution.
3. In general the larger the solvent accessible protein surface is, the more identifiable halide sites can be expected. However, the number of highly occupied sites may depend on several factors, such as the concentration of the halide (and other salts) and the chemical composition and pH of the solution, the isoelectric point of the protein, the presence of the positively charged residues as well as hydrophobic patches at the protein surface, and others. It is therefore not possible to predict how many halide sites can be expected in each individual case.
4. The comparisons of putative sites from separate direct methods solutions have to take into account the space group symmetry operations as well as the possible origin shifts and inversion of the enantiomer.

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