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ORIGINAL RESEARCH

Advantages of time-resolved difference X-ray solution scattering curves in analyzing solute molecular structure

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Abstract Time-resolved X-ray solution scattering provides a powerful method for investigating reaction dynamics in the solution phase. Since X-rays scatter from all atoms in the solution sample, the scattering intensity is contributed from not only the solute but also the solvent and the solute–solvent cross terms. For a typical concentration the solvent molecules outnumber the solute molecules and thus the relative sensitivity of the scattering intensity to the solute structure is extremely low. To increase the structural sensitivity to the solute and to extract only the signal from structural changes, time-resolved difference scattering signal is obtained by subtracting the original raw scattering curve at a negative reference time delay from that at a positive time delay. Here we show and emphasize that time-resolved difference X-ray scattering curves generally exhibit higher structural sensitivity to the solute molecular structure and lower influence from experimental background and imperfection of theory than original raw scattering curves. These characteristics justify the validity of fitting models to difference curves to obtain transient structural information even when the magnitude of the time-resolved difference curves is smaller than the discrepancy between the theory and experiment for the original scattering curve. We considered small molecules and proteins in solution probed by time-resolved X-ray solution scattering.

Keywords Time X-ray scattering · Time-resolved · Solute · Liquidography · Solution scattering

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Introduction

Time-resolved scattering with either short-pulse X-rays [1–18] or electrons [19–28] offers a new and powerful molecular probe that is complementary to time-resolved optical spectroscopy [29–44], for monitoring structural changes of molecules in the course of a reaction. In a typical experiment [7–11, 13–16, 19, 22–27, 45–48], a sample containing the molecules of interest, is irradiated by an ultrashort optical pulse to initiate a reaction, and after a well-defined time delay, ultrashort X-ray/electron pulses are sent to the sample undergoing reaction processes and the scattered X-rays/electrons, which carry the structural information about the molecules at that time delay, are detected. The advantage of the scattering method includes the fact that the scattering pattern or curve can be quantitatively calculated from atomic coordinates of molecules, thereby providing a direct and quantitative link connecting the observed signal to molecular structure [8–11, 13–16, 19, 22–27, 45–48]. The experimental scattering curve from a sample of interest can be fitted against a theoretical model until a satisfactory agreement between experiment and theory is reached, yielding structural information about the species. In time-resolved scattering studies [8–11, 13–16, 19, 22–27, 45–48], the difference between the total scattering curve at a specific time delay and that at a reference delay, usually a negative delay, is obtained and used for the structural refinement. The standard approach is to fit the experimental difference curves to theoretical difference curves from candidate molecular structures, thereby yielding useful information about the structure of reaction intermediates.

Due to error propagation, errors associated with measurement are slightly (by a factor of square root of two) increased in a difference curve compared with an original

curve, and thus a difference curve has an inferior signal to noise ratio. However, there are many advantages of working with difference curves [23–25] that compensate the signal to noise issue. These include (i) the unchanged intensity such as the atomic scattering that contributes to the majority of the original raw scattering is removed, bringing out only the signal from structural changes, (ii) intrinsic systematic error in the detection system is greatly reduced or eliminated, and (iii) the relative contribution of the transient species compared with the parent species is greatly enhanced whereas the original raw data contains a relatively small fraction of transient species and majority of parent species. These advantages have been well-demonstrated for the case of gas-phase electron diffraction where the species of interest is isolated.

By contrast in the solution phase the solute molecule is surrounded by many solvent molecules, which often outnumber the solute. Thus, the solution scattering intensity is contributed not only from the solute but also from the solvent and the solute–solvent cross terms, and the relative sensitivity of the scattering intensity to the solute structure is extremely low. Often it is impossible to refine the molecular structure of the solute by using the static X-ray scattering data. On the contrary, the difference scattering data can be used to provide useful information about the solute structure. Our experience shows that time-resolved difference scattering curves usually provide superior agreement between theory and experiment compared to the original raw scattering curves. More seriously, the discrepancy between the theory and experiment for the original scattering curve is sometimes comparable to the magnitude of time-resolved difference scattering curves. This raises serious doubt about the validity of fitting difference curves to obtain transient structural information. Here we show that time-resolved difference scattering curves are more sensitive to structure or structural changes, than the original raw scattering curves, mainly because a large fraction of the total signal that is not sensitive to the molecular structure is removed in the difference curve. Together with this enhanced structural sensitivity, experimental background whose exact nature and functional form is generally difficult to obtain and therefore complicates the analysis of original curves, are essentially eliminated in difference curves. In addition, the theory used to calculate the curve may not be perfect to account for the experimental condition, but the effect of the shortcoming of theory can be much less in the difference curve than the original curve because the experimental contribution that cannot be explained by the limited theory can be cancelled out in the difference curve. These considerations justify the validity of fitting difference curves to obtain transient structural information even when the discrepancy between theory and experiment for the original scattering curve is sometimes comparable to the magnitude of the time-resolved difference scattering curves. We considered structural sensitivity for small molecules in the solution phase by solution X-ray scattering, and protein molecules in solution by combined wide-angle X-ray scattering (WAXS).

Structural sensitivity

I_2 dissolved in methanol investigated by solution X-ray scattering

In this case, the solute molecule (I_2) is no longer isolated, but surrounded by solvent molecules (for example, methanol). Therefore, the total scattering intensity (I_{Tot}) is a sum of the scattering from the solute alone ($I_{\mathrm{Solute\text{-}only}}$), the solute–solvent cross term ($I_{\mathrm{Solute–solvent}}$), and the scattering from solvent alone ($I_{\mathrm{Solvent\text{-}only}}$). In reality, a certain background (I_{bkg}) due to imperfect experimental conditions such as the non-linear detector response and scattering from air and the sample holder that has been imperfectly subtracted need to be considered:

$$I_{\mathrm{Tot}} = I_{\mathrm{Solute-only}} + I_{\mathrm{Solute-solvent}} + I_{\mathrm{Solvent-only}} + I_{\mathrm{bkg}} \qquad (1)$$

In most cases, $I_{\mathrm{Solute\text{-}only}}$ and $I_{\mathrm{Solute–solvent}}$ are minor components and $I_{\mathrm{Solvent\text{-}only}}$ dominates the signal. To assess the structural sensitivity, we considered an I_2 molecule in methanol with I···I distance of 2.7 Å and its I_{Tot} as a reference. Details about calculating or estimating each component is discussed in our earlier publications. Then we generated I_{Tot} curves for various I···I distances. We simulated a curve for 20 mM to consider a typical solute concentration. Figure 1a shows a comparison of X-ray diffraction intensities for the reference molecule and a perturbed molecule with $\Delta r = 0.3$ Å as an example. Figure 1b shows a comparison of $\Delta I_{\mathrm{Tot}}(q)$ for the reference molecule and a perturbed molecule with $\Delta r = 0.3$ Å as an example. The degree of deviation was measured by the R^2 factor defined by

$$R^2 = \sum (f - f_{\mathrm{ref}})^2 \Big/ \sum (f_{\mathrm{ref}})^2 \qquad (2)$$

where f is I_{Tot} for the original curve and ΔI_{Tot} for the difference curve, and ref is to indicate the reference curve. The higher R^2 value means higher discrepancy between the reference curve and the curve from a perturbed structure. The slope of the R^2 curve is therefore proportional to the sensitivity of the diffraction data to the molecular structure. As shown in Fig. 1d, the structural perturbation reflected as Δr affects the R^2 factors very little, indicating that the total signal is extremely insensitive to the structure of the solute. This is because $I_{\mathrm{Solvent\text{-}only}}$ dominates. Then we considered a favorable situation where we can subtract $I_{\mathrm{Solvent\text{-}only}}$

perfectly without introducing systematic errors, which is hard to accomplish in reality. In this case, the structural sensitivity is much enhanced as indicated by the increased slope in Fig. 1.

For the time-resolved experiment, we consider a difference scattering intensity (ΔI_{Tot}). As mentioned, unknown errors associated with I_{bkg} is removed or at least greatly reduced in the difference ($\Delta I_{\mathrm{bkg}} = 0$).

$$\Delta I_{\mathrm{Tot}} = \Delta I_{\mathrm{Solute-only}} + \Delta I_{\mathrm{Solute-solvent}} + \Delta I_{\mathrm{Solvent-only}} \quad (3)$$

The last term ($\Delta I_{\mathrm{Solvent\text{-}only}}$) is very sensitive to the thermodynamic state of the bulk solvent, which may change during a chemical reaction due to energy transfer from light-absorbing solute molecules to the surrounding solvent molecules and the following relaxation to equilibrium with the environment around the scattering volume. Now the solute-related terms ($\Delta I_{\mathrm{Solute\text{-}only}}$ and $\Delta I_{\mathrm{Solute-solvent}}$) are major components. Especially in large scattering angles, their contribution is at least comparable to the other two terms or dominates the whole signal depending on the nature of the solvent.

To assess the structural sensitivity of ΔI_{Tot}, we generated a reference curve by considering the dissociation of I_2 into two iodine atoms in methanol. Then, we generated ΔI_{Tot} curves for various I···I distances. A solute concentration of 20 mM was used and only 10% of the initial solute is assumed to undergo fragmentation to represent a typical case.

Figure 1d shows the R^2 factors as a function of Δr. It is evident that the R^2 factor of ΔI_{Tot} depends on the Δr more strongly than that of I_{Tot} and that of solvent-subtracted I_{Tot}, indicating that the difference curve is much more sensitive to the structural parameters than the original curve is. The same is also evident in Fig. 1c where the difference between the reference curve and the perturbed curve for $I_{\mathrm{Tot}}(q)$ and $\Delta I_{\mathrm{Tot}}(q)$ are compared.

If the original scattering curve contains no experimental background (I_{bkg}), in principle, it should be possible to extract accurate structural parameters by fitting the experimental curve with a theoretical curve noting that the experimental noise is much reduced by averaging. However, the exact functional form of I_{bkg} is generally unknown and therefore a certain error is unavoidably introduced in the process of subtracting background from I_{Tot} to bring out I_{Mol}, the original curve is more vulnerable to this error associated with I_{bkg}. By contrast, this

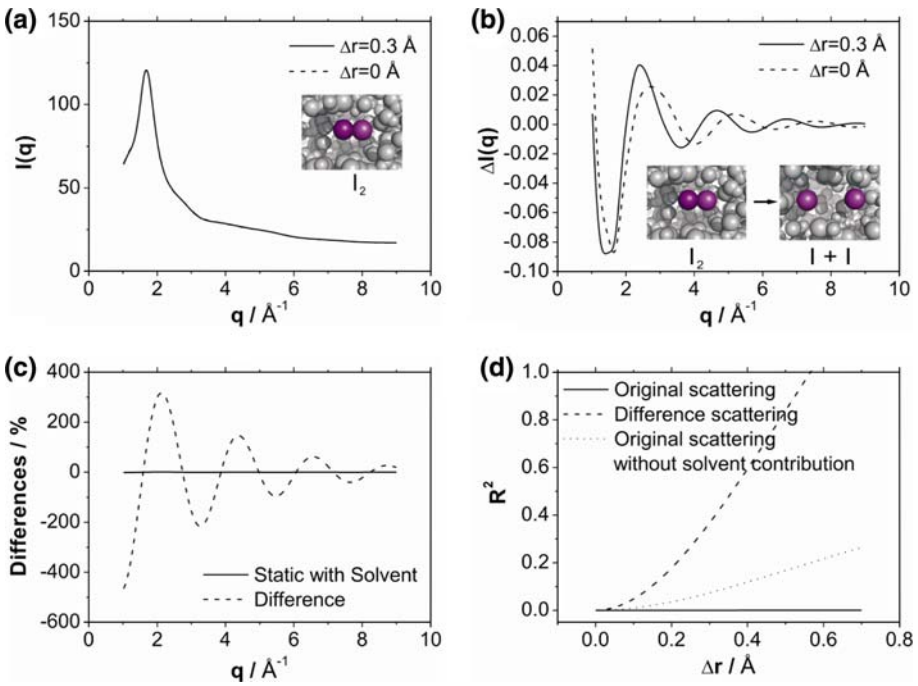

Fig. 1 **a** Liquid-phase X-ray scattering intensities, $I(q)$, of the reference I_2 molecule with an I···I distance of 2.7 Å ($\Delta r = 0$ Å, *dashed line*) and the perturbed I_2 molecule ($\Delta r = 0.3$ Å, *solid line*) in methanol. **b** The difference diffraction intensities, $\Delta I(q)$, for the dissociation reaction of I_2 in methanol ($I_2 \rightarrow I + I$). The *solid line* is for the perturbed I_2 molecule whose Δr is 0.3 Å, and the *dashed line* is for the reference I_2 molecule. **c** The difference between the reference curve and the perturbed curve for the case of **a** (in *solid line*) and **b** (in *dashed line*). Each curve is normalized by the average absolute value of $I_{\mathrm{Tot}}(q)$ for **a** and $\Delta I_{\mathrm{Tot}}(q)$ for **b**, respectively. The enhanced structural sensitivity of $\Delta I_{\mathrm{Tot}}(q)$ over $I_{\mathrm{Tot}}(q)$ is evident. **d** R^2 factors for $I_{\mathrm{Tot}}(q)$ (in *solid line*) and $\Delta I_{\mathrm{Tot}}(q)$ (in *dashed line*) as a function of Δr between the reference I···I distance and the perturbed distance. The slope of the R^2 curve is proportional to the sensitivity of the diffraction data to the molecular structure, indicating that the structural sensitivity of $\Delta I_{\mathrm{Tot}}(q)$ is much better than $I_{\mathrm{Tot}}(q)$. When the solvent contribution is ignored from $I_{\mathrm{Tot}}(q)$, the slope of the R^2 curve is enhanced (*dotted line*)

kind of error associated with I_{bkg} is removed or at least greatly reduced in the difference curve because I_{bkg} can be subtracted out even if the exact functional form is not known.

Hemoglobin in solution investigated by WAXS

As the size of the solute molecule increases, for example, up to that of protein molecules, small angle scattering intensities becomes important due to the reciprocal relationship between the internuclear distance and the scattering angle. Solution X-ray scattering of protein has been widely used to determine overall protein structure under various physiological conditions and conformational changes due to the variation of external parameters such as pH, temperature, and denaturant concentration [49–54]. Usually the SAXS region provides overall structural features such as size and shape, and WAXS probes distance correlations on shorter length scales and therefore contains rich information of detailed fine structures such as the fold of secondary structures.

In a typical static SAXS/WAXS experiment, the scattering intensity of a pure buffer without protein is measured and subtracted from the scattering intensity of a protein solution. In a sense this removes the contribution from the solvent-only term in Eq. 1. The total scattering intensity can be given by a sum of three basic scattering functions and some background.

$$I_{\mathrm{Tot}} = \overline{\rho\rho} I_{\mathrm{F}} + \overline{\rho} I_{\mathrm{FI}} + I_{\mathrm{I}} + I_{\mathrm{bkg}} \tag{4}$$

where $\overline{\rho}$ is the mean excess electron density (the difference between the mean electron density of the particle and the electron density of the solvent), and I_{F}, I_{FI}, and I_{I} are the scattering intensity due the shape of the particle, the cross term, and the internal structure of the particle, respectively. I_{bkg} is a certain background due to imperfect experimental conditions such as the non-linear response of the detector system and scattering from the buffer and sample holder that has been imperfectly subtracted. For a typical TR-WAXS experiment, a much higher concentration is used and therefore inter-particle interference that can be usually ignored in normal SAXS measurement may contribute. In that case, the inter-particle interference can also contribute to I_{bkg}.

If we consider a difference intensity,

$$\Delta I_{\mathrm{Tot}} = \Delta(\overline{\rho\rho} I_{\mathrm{F}}) + \Delta(\overline{\rho} I_{\mathrm{FI}}) + \Delta I_{\mathrm{I}} \tag{5}$$

the first term is relatively small and the last term that is most sensitive to the internal structure of the particle, becomes comparable to the first term. As previously mentioned, unknown errors associated with I_{bkg} are removed or at least greatly reduced in the difference curve ($\Delta I_{bkg} = 0$).

To compare the structural sensitivities of a static WAXS curve and a time-resolved difference WAXS curve, we considered the hemoglobin (Hb) protein molecule, a model system that has long served as a paradigm for understanding allosteric regulation and conformational dynamics in proteins. Hb is a tetrameric 64 kD protein that consists of two α and two β subunits held together by non-covalent interactions. Each of the four subunits contains a heme prosthetic group whose Fe(II) reversibly binds gaseous molecules such as O_2, CO, and NO. X-ray crystallography has shown that the quaternary structure of fully oxygenated Hb (R state) is distinct from that in fully deoxygenated Hb (T state), with the $\alpha\beta$ dimers rotated approximately 15° relative to each other.

To assess the structural sensitivity of static WAXS curves, we used a crystallographic structure R2 (pdb code: 1BBB) as a reference. Various other structurally perturbed coordinates were generated by Molecular Dynamics (MD) simulations. The simulated annealing method in Xplor-NIH [55] generated these structures from the original R2 structure. For each structure, we used the CRYSOL [56] program to calculate the corresponding WAXS curve. For difference WAXS curves, we generated a reference curve by considering the quaternary structural transition from T to R2. We used a crystallographic structure for T (pdb code: 2HHB) and we generated ΔI_{Tot} curves for various R2 structures generated by MD simulations. Figure 2d shows the dependence of the R^2 factors on the RMSD. It is clear that the structural sensitivity of difference WAXS curves is much stronger than that of static WAXS curves. Figure 2a shows a comparison of the reference static WAXS curve and a structurally perturbed WAXS curve with the RMSD value of around 0.6 Å. Their difference shown in Fig. 2c is on average within 5% of the mean of the absolute value of the reference WAXS curve. Figure 2b shows a similar comparison for the difference WAXS curves. In this case, the difference shown in Fig. 2c is on average as much as 57% of the mean of absolute value of the reference difference WAXS curve. In summary our result shows that the difference WAXS curve is much more sensitive to subtle structural changes than the original static WAXS curve.

Other advantages of difference WAXS curves over static WAXS curves merit attention. In actual static WAXS experiments, the protein solution is contained in a sample holder. To extract the scattering from the protein only, the scattering intensity from the sample holder alone and the sample holder containing only the buffer solution need to be separately measured and properly scaled and subtracted from the scattering of the sample holder containing the protein solution. If some error is introduced in this process, its impact can be substantial.

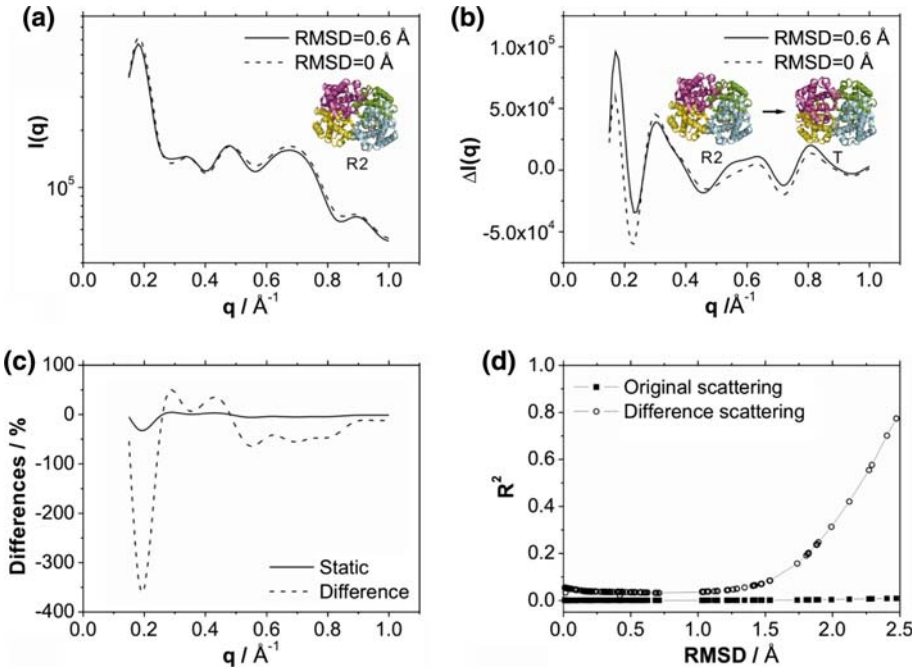

Fig. 2 **a** WAXS intensities from the hemoglobin models for the original scattering intensities from the R2 structure of hemoglobin (pdb code: 1BBB, *dashed line*) and the perturbed structure (RMSD = 0.6 Å, *solid line*). **b** The difference scattering intensities generated by the subtraction of the scattering intensity of R2 or its perturbed structure from hemoglobin T form (pdb code: 2HHB). The curve using R2 structure is shown as the *dashed line*, and the curve using the perturbed structure is shown as the *solid line*. **c** The difference between the reference curve and the perturbed curve for the case of **a** (in *solid line*) and **b** (in *dashed line*). Each curve is normalized by the average of the absolute value of $I_{\mathrm{Tot}}(q)$ for **a** and $\Delta I_{\mathrm{Tot}}(q)$ for **b**, respectively. The enhanced structural sensitivity of $\Delta I_{\mathrm{Tot}}(q)$ over $I_{\mathrm{Tot}}(q)$ is evident. **d** R^2 factor for WAXS intensities as a function of RMSD between the reference structure and the perturbed structure. R^2 factor for $I_{\mathrm{Tot}}(q)$ is shown as *black squares*, and that for $\Delta I_{\mathrm{Tot}}(q)$ is shown as *open circles*. The slope of the R^2 curve is proportional to the sensitivity of the diffraction data to the molecular structure, indicating that the structural sensitivity of $\Delta I_{\mathrm{Tot}}(q)$ is much better than $I_{\mathrm{Tot}}(q)$

However, in time-resolved WAXS experiment, the normalization and subtraction process is much simplified since the scattering from the sample holder containing the protein sample can be used to directly obtain the difference WAXS curve by overriding the usual process used in the static WAXS data treatment. More importantly the usual calculation approach (for example CRYSOL [56] program) employs the continuum-solvent model where uniform electrons densities with proper shape indicators are used for the excluded solvent region and the solvation layer for the sake of simplicity and fast calculation. Recently it has been shown that the continuum-solvent description introduces errors because the density fluctuations of the bulk solvent (excluded volume) and the solvation layer around the uniform electron densities are omitted, and thus the explicit description of the solvent electron densities (explicit-solvent model) is necessary [57]. The error caused by the continuum model is greatly reduced in the difference WAXS curve [58]. In addition, the effect of inter-protein interference for highly concentrated sample, which complicates the data analysis, is also largely reduced in the difference curve.

Conclusions

We have shown that difference scattering curves generally exhibit much higher structural sensitivity to the solute structure than original scattering curves because a large fraction of the original scattering curve that is insensitive to molecular structure and is contributed by the solvent, is removed in the difference curve. In addition to this enhanced structural sensitivity, experimental background whose exact nature and functional form is generally difficult to obtain and therefore complicates the analysis of original curves, are essentially eliminated in difference curves and the effect of the shortcoming of theory can be much less in the difference curve than the original curve. These facts justify the validity of fitting difference curves to obtain transient structural information even when the discrepancy between theory and experiment for the original scattering curve is sometimes comparable to the magnitude of the time-resolved difference scattering curves.

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