Can infrared spectroscopy provide information on protein–protein interactions?

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Abstract
For most biophysical techniques, characterization of protein–protein interactions is challenging; this is especially true with methods that rely on a physical phenomenon that is common to both of the interacting proteins. Thus, for example, in IR spectroscopy, the carbonyl vibration (1600–1700 cm−1) associated with the amide bonds from both of the interacting proteins will overlap extensively, making the interpretation of spectral changes very complicated. Isotope-edited infrared spectroscopy, where one of the interacting proteins is uniformly labelled with 13C or 13C,15N has been introduced as a solution to this problem, enabling the study of protein–protein interactions using IR spectroscopy. The large shift of the amide I band (approx. 45 cm−1 towards lower frequency) upon 13C labelling of one of the proteins reveals the amide I band of the unlabelled protein, enabling it to be used as a probe for monitoring conformational changes. With site-specific isotopic labelling, structural resolution at the level of individual amino acid residues can be achieved. Furthermore, the ability to record IR spectra of proteins in diverse environments means that isotope-edited IR spectroscopy can be used to structurally characterize difficult systems such as protein–protein complexes bound to membranes or large insoluble peptide/protein aggregates. In the present article, examples of application of isotope-edited IR spectroscopy for studying protein–protein interactions are provided.

Protein structure analysis using IR spectroscopy
Interaction between different proteins is key to many vital biochemical processes in living systems. Unfortunately, our understanding of such interactions remains limited, mainly due to a lack of suitable techniques that can be used to study protein–protein interaction. NMR spectroscopy and X-ray crystallography are by far the most powerful techniques for such studies, providing structural data at the atomic level. However, both of these techniques are restricted by various factors such as protein solubility, size or environment. Thus, for example, X-ray crystallography requires proteins in the crystalline state, which is not easy in many cases, such as with membrane proteins. NMR spectroscopy is often limited to proteins that are soluble and not too large (less than 40 kDa). Hence there is a need to identify other techniques that can provide useful structural information on protein–protein interactions involving systems that are beyond the reach of NMR and X-ray crystallography. One such technique that is potentially powerful is FTIR (Fourier-transform IR) spectroscopy. Historically, IR spectroscopy is one of the earliest techniques to be used for protein structure analysis (for a review, see [1]) and its usefulness in this area is summarized in the present article (see below). IR spectroscopy is based on vibrations of atoms within molecules. Those vibrations that result in a change in the net dipole moment of the molecule will appear as peaks in an IR spectrum. For protein analysis, the vibration of the peptide bond has been most productively used for characterization of protein secondary structure. The amide I band (see Figure 1), associated mainly with C=O stretching vibration of the peptide bond, can be related to the secondary structure of the protein. The amide I band is near 1650 cm−1 for α-helical structure, whereas β-sheet structure occurs near 1630–1640 cm−1. Analysis of a large number of proteins and peptides has led to a general consensus regarding the position of the amide I band and the type of protein secondary structure (see Table 1). Nevertheless, more work needs to be done in this area to further improve our understanding of factors that govern changes in amide band positions as a function of protein structure. The amide II band, which arises primarily from N–H bending vibration of the peptide bond, is particularly useful for monitoring hydrogen–deuterium exchange and changes in hydrogen-bonding [1,2].

What are the advantages of IR spectroscopy for protein–protein interaction studies?
The particular strength of IR spectroscopy lies in its ability to probe protein structure in diverse environments [2], ranging from aqueous solution, organic solvents, suspensions (in micelles and lipid bilayers), solid state (thin films, powders, crystals) and even the gaseous phase. There are few techniques that can match IR spectroscopy in being able to provide...
Table 1 | Relationship between amide I band position and protein secondary structure
The assignments given are based on general agreement from work published in the literature.

<table>
<thead>
<tr>
<th>Secondary structure</th>
<th>Wave number in $^{1}$H$_2$O</th>
<th>Wave number in $^{2}$H$_2$O</th>
</tr>
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<tr>
<td>α-Helix</td>
<td>1648–1660 cm$^{-1}$</td>
<td>1642–1660 cm$^{-1}$</td>
</tr>
<tr>
<td>β-Sheet</td>
<td>1620–1640 cm$^{-1}$</td>
<td>1615–1640 cm$^{-1}$</td>
</tr>
<tr>
<td></td>
<td>1670–1695 cm$^{-1}$</td>
<td>1670–1694 cm$^{-1}$</td>
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<tr>
<td>Turns</td>
<td>1620–1640 cm$^{-1}$</td>
<td>1653–1694 cm$^{-1}$</td>
</tr>
<tr>
<td></td>
<td>1650–1695 cm$^{-1}$</td>
<td></td>
</tr>
<tr>
<td>Unordered</td>
<td>1640–1657 cm$^{-1}$</td>
<td>1639–1654 cm$^{-1}$</td>
</tr>
<tr>
<td></td>
<td>1660–1670 cm$^{-1}$</td>
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Figure 1 | FTIR spectrum of papain from papaya latex in aqueous ($H_2O$) solution
(A) Absorbance spectrum and (B) second-derivative spectrum. The secondary structure of papain consists of a mixture of α-helices and β-sheets, evident from the strong bands at 1650 and 1633 cm$^{-1}$ respectively.

structural data in such a wide variety of environments. Unlike many other biophysical techniques, it is not restricted by the size of the protein molecule or its solubility/insolubility or aggregation state. Thus the first evidence for β-sheet structure in the abnormal form of the prion protein was derived from IR spectroscopy. The insolubility of the prion protein aggregates and the inability to produce crystals prevented the utilization of both NMR spectroscopy and X-ray crystallography respectively.

Potentially, IR spectroscopy can measure very small changes in molecular structure, such as those associated with subtle alterations in bond length, which are beyond the reach of NMR and X-ray crystallography. However, for these small changes in molecular structure to be observed, it is necessary to utilize specialized techniques and tools. The amide I band (see Figure 1A) is a broad band consisting of absorbance from all the amide bonds within the protein molecule. The vibrational frequency of each of these bonds will depend on their hydrogen-bonding patterns and type of secondary structure. Owing to the broad band width, there is extensive overlap between the groups which makes it difficult to distinguish between different secondary structures, let alone individual amide bonds. This is where one can benefit from the utilization of isotopic labelling. Thus, if individual peptide bonds were isotopically labelled, it will be possible to separate the absorbance of a specific peptide bond from the other peptide groups. In this way, changes in the structure of the peptide associated with this single group can be monitored in a very large protein consisting of hundreds of amino acids. Visualization of this single peak can be achieved through the use of difference spectroscopy or so-called resolution enhancement techniques such as second-derivative and deconvolution methods. The absorbance and second-derivative of papain which consists of a mixture of helical and β-sheet structure are shown in Figure 1(A) and Figure 1(B) respectively. Second-derivative and deconvolution methods do not alter the instrumental resolutions which are set during the recording of the spectra. However, they are based on band-narrowing methods that aid in the separation of the peaks through application of mathematical and computational techniques. These are now widely used and are part of software provided by IR spectrometer manufacturers. Using these methods, it is possible to monitor subtle changes in IR peaks as a function of various factors such as changes in pH, temperature or interaction with ligands. Unfortunately, it is still not possible to obtain information at the individual residue level using such procedures. However, thanks to development in chemical synthesis and molecular biology, it is now possible to isotopically label proteins at specific residues and then carry out IR spectroscopic measurements.

Listed below are a few of the advantages offered by IR spectroscopy for studying protein–protein interactions and the types of information that can be obtained.

(i) Protein–protein complexes in diverse environments. Probably the greatest advantage of IR spectroscopy over many other techniques is its versatility in being accessible for analysis of proteins in a diverse range of environments. This is particularly important, since in living systems, as well as in industrial applications, it is necessary to know how a protein will behave structurally and functionally as a consequence of changing environment. Thus it is possible to analyse protein structure in solution (aqueous and non-aqueous), in the solid state (crystals, powder, and thin film) or in suspensions (membranes, aggregates).
Figure 2 shows the IR spectrum of a protein in lipid membranes.

(ii) Enables analysis of insoluble protein–protein aggregates. It is possible to study the interaction between a peptide or protein in an aggregated state with another protein. This is often impossible using techniques such as X-ray crystallography or NMR spectroscopy.

(iii) Does not require a probe molecule. Unlike some spectroscopic techniques, IR spectroscopy does not require the attachment of a probe molecule for obtaining structural information. This avoids the need to employ molecules that may potentially perturb the interaction between two proteins.

(iv) Non-destructive. Protein–protein complexes are not damaged by conventional IR light and the sample can be reused. This is not the case with some techniques where the proteins can be damaged and cannot be reused.

(v) Secondary structure. From the analysis of the amide bands, especially the amide I band, it is possible to probe the secondary structure of a protein or peptide while it is engaged in an interaction with another peptide or protein.

(vi) Amino acid side-chain structure. Changes in amino acid side chains can be revealed most effectively using difference spectroscopy, especially with site-specific isotopic labelling of a specific amino acid residue. Difference spectroscopy (Figure 5) is a powerful tool, as the subtraction of a protein spectrum in state A from that in state B results in cancellation of the peaks that do not undergo a conformational change. The resultant difference spectrum only contains peaks from those groups that have undergone a structural change. Through this approach, IR spectroscopy can yield structural changes involving protein–protein interaction at the level of individual residues.

(vii) Protein dynamics. Hydrogen–deuterium exchange within the peptide group and shifts in band position associated with changes in environment can be analysed. Hydrogen–deuterium exchange of one protein while complexed to another protein can be monitored. Furthermore,
the hydrogen–deuterium exchange of specific secondary-structural elements can be monitored, whereas in a protein–protein complex, with site specific labelling, the hydrogen–deuterium exchange of a specific residue can be monitored.

(viii) Protein stability. Stability of proteins under the influence of temperature, pH, etc. With isotopic labelling, the stability of protein while complexed to another protein can be proved. As can be seen from Figure 4, the thermal stability of an Ig domain has been monitored while it is participating in an interaction with G-CSF (granulocyte colony-stimulating factor). It was found that, while in a complex, the melting transition ($T_m$) of the $\beta$-structure in the Ig domain and the $\alpha$-helical structure in G-CSF increases.

(ix) Protein orientation. Changes in the orientation of a protein can be determined through polarized IR spectroscopy. With isotopic labelling, orientation of specific residues can be monitored.

Studying protein–protein interaction with and without isotopic labelling

Studying interaction between two molecules that are not dominated by identical types of vibrational groups is straightforward. Thus, for example, interaction between proteins and lipids have been extensively studied by taking advantage of the fact the key protein band, associated with peptide bond, occurs between 1700 and 1600 cm$^{-1}$ and is well separated from the important lipid vibrations associated with the acyl hydrocarbon chains (2800–2900 cm$^{-1}$), the lipid ester vibration (1720–1740 cm$^{-1}$) and the phosphate headgroups. However, when studying interaction between two molecules sharing the same type of vibrational groups, such as that between a protein and another protein or between a lipid and another lipid molecule, there is extensive overlap between the common vibrational groups, making it difficult to distinguish one protein from another protein. It may be possible to avoid the effect of the overlapping signals if the concentration of one of the interacting molecules is sufficiently low so that it does not significantly affect the signal from the other protein. However, in many situations, this is not possible as a similar concentration of the two molecules is necessary for an interaction to occur. In such situations, there are two alternatives: (i) to use computational and mathematical procedures to identify whether a conformational change results from the interaction. This approach does not involve isotopic labelling; or (ii) to isotopically label one of the

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**Figure 3** | FTIR second-derivative spectra [7] of the isolated receptor Ig domain (top panel), G-CSF–Ig complex (middle panel) and the isotope-labelled ($^{13}$C,$^{15}$N) G-CSF–Ig complex (bottom panel).

**Figure 4** | Studying thermostability of individual proteins in protein–protein complexes [7]

FTIR second-derivative spectra of the $^{13}$C,$^{15}$N G-CSF–Ig complex recorded at various temperatures (from 20 to 90°C).
Isotope-edited FTIR spectroscopy for studying the interaction between rhodopsin and a peptide corresponding to its effector G-protein (transducin)

The top panel shows the spectrum in the presence of unlabelled and \(^{13}\text{C},^{15}\text{N}\)-labelled peptide. The middle panel represents the double-difference spectrum which reveals the shifts in the amide I and amide II regions resulting from the isotopic labelling. The component spectra of the receptor and the peptide were generated through deconvolution of the isotopic shifts [8].

Interacting molecules, or at least part of it, so that its frequency is shifted away from the bands of the other molecule which will enable the structural study of both proteins.

The first approach (without isotopic labelling) involves recording the spectrum of the individual proteins and then digitally adding them to produce a single spectrum which can then be compared with the spectrum obtained for the proteins while they are actually interacting with each other. If there are differences between the synthetic spectrum (for example, where the spectrum of protein A is added to the spectrum of protein B) and the spectrum of the actual complex between protein A and B, then it would be indicative of some type of interaction between the two proteins. However, it would not be straightforward to identify which of the two proteins have undergone a change during the interaction. In order to overcome this problem, Jung and co-workers have proposed a new method that utilizes computational and mathematical procedures to identify spectral changes in the spectra of protein–protein complexes [3]. The process involves combining FTIR spectroscopy with protein titration measurements and then carrying out principal component analysis of the IR spectrum of the protein complex in the amide I and amide II region [3]. The authors used this approach to study the interaction between the haem domain with the FMN domain of a bacterial mono-oxygenase cytochrome P450BM-3 (CYP102A1). The principal component analysis revealed that the interaction results in alterations in protein secondary structure and amino acid side chains. The advantage of this approach is that there is no need for expensive labelling of proteins. However, the disadvantage is that it lacks specificity, since one cannot unequivocally determine which of the two interacting proteins have undergone the structural change.

Herein lies the advantage of isotope-edited IR spectroscopy, since the amide I band of one of the interacting proteins is unmasked, and changes in its band width, band frequency and band position can be readily monitored without having to rely on theoretical calculations.

The substitution of a heavier atom (e.g. deuterium) for a lighter atom (e.g. hydrogen) results in a change in mass that will have an effect on the vibration of a particular bond. Thus, for example, the IR spectra of \(^{1}\text{H}_{2}\text{O}\) and \(^{2}\text{H}_{2}\text{O}\) are very different due to this mass effect. The H-O-H stretching vibration is located at approx. 1650 cm\(^{-1}\), overlapping with the protein amide I band, whereas the \(^{2}\text{H}-\text{O}-^{2}\text{H}\) vibration occurs at 1215 cm\(^{-1}\). In the past, IR spectroscopy had to be conducted in the solid state because of the strong absorbance of the H-O-H bending vibration. However, replacing \(^{1}\text{H}_{2}\text{O}\) with \(^{2}\text{H}_{2}\text{O}\) and the associated isotopic shift results in a window in the region between 1700 and 1600 cm\(^{-1}\). This enables the analysis of the amide I band of protein in an aqueous environment (\(^{2}\text{H}_{2}\text{O}\)). Thanks to digitization of spectral data, it is now possible to also record spectra of protein in \(^{1}\text{H}_{2}\text{O}\) as the H-O-H band can be digitally subtracted to reveal the underlying amide I band of proteins. The concept of isotopic shift has also been used for the analysis of proteins where specific amino acid side chain or peptide backbone has been labelled with \(^{2}\text{H},^{13}\text{C},^{15}\text{N}\) and \(^{18}\text{O}\). Measurement of hydrogen–deuterium exchange in proteins, where the replacement of the amide proton with deuterium results in a shift of the amide II band from approx. 1550 cm\(^{-1}\) to 1450 cm\(^{-1}\), has been successfully used for studying protein conformation and dynamics [1,2].

Isotopic labelling has been useful for specifying the role of individual residues and also for simplifying spectral interpretation. Although many studies have been reported on proteins and peptides, with isotopic labels introduced at specific sites, it was not until 1992 that the potential of using uniformly labelled proteins for studying protein–protein interactions using IR spectroscopy was first introduced [4]. This advance came at a time when molecular biological methods were available for uniform labelling of proteins. Currently, bacterial and eukaryotic expression systems can
be used to overproduce labelled proteins, making it easier to carry out isotope-edited spectroscopic studies. More recently, cell-free systems and strategies are being developed for production of labelled proteins.

Of the different types of isotopic labelling, ¹³C labelling is most useful in protein–protein interaction studies, as this results in a large shift of the amide I band which arises principally from C=O stretching vibration [4]. Thus replacement of the ¹²C with ¹³C, and the associated mass change, has a large effect on the stretching vibration of the carbonyl bond. Approx. 45 cm⁻¹ shift of the amide I band towards a lower frequency is associated with the ¹³C labelling (see Figure 2). This shift is sufficient to reveal the amide I band of the unlabelled protein (1700–1620 cm⁻¹) when studying the interaction between two proteins, one uniformly ¹³C labelled and the other left unlabelled.

**Examples of isotope-edited FTIR spectroscopy**

Figure 2 shows the absorbance spectrum of a protein before and after it has been uniformly ¹³C,¹⁵N,²H-labelled. The large shift of approx. 45 cm⁻¹ associated with the ¹³C labelling is clearly visible. The amide I band of the unlabelled protein is now well separated from the amide I band of the labelled protein. This makes it very easy to detect whether there are any changes in the structure of the unlabelled protein through the analysis of the amide I band. It is also possible to visualise the amide I band of the labelled protein; however, there is some overlap with the amide II vibration of the labelled protein, which makes it a bit more complicated.

Figure 2 presents the FTIR spectrum (P.I. Haris, V. Réat and A. Milon, unpublished work) of a triple (¹³C,¹⁵N,²H) labelled protein in DHPC (dihexanoyl phosphatidylcholine) micelles in ²H₂O. It corresponds to the spectrum of the outer membrane protein A from *Klebsiella pneumoniae* (KpOmpA). The protein has a β-barrel structure according to NMR spectroscopy [5]. The IR amide I maximum near 1630 cm⁻¹ is consistent with the NMR results. For comparison, the IR spectrum of the unlabelled and double-labelled (¹³C,¹⁵N) protein are also shown. The key difference between the double-labelled (¹³C,¹⁵N) and unlabelled protein is the large shift in the amide I band frequency from approx. 1630 cm⁻¹ to approx. 1585 cm⁻¹. Only slight changes occur after triple labelling (¹³C,¹⁵N,²H), although these changes can be useful for monitoring subtle changes in structure. Since the C=O vibration of the peptide bond is linked directly to the nitrogen within the peptide bond structure, the labelling of the nitrogen atom has a slight effect on the position of the amide band. It shifts slightly further towards lower frequency [4]. However, labelling of the protein with ²H is not directly associated with the peptide bond, as the hydrogen atom within the peptide bond is exchangeable and can be either protonated or deuterated. Although the deuteration of the hydrogen atom in the peptide bond will lead to a shift towards lower frequency, the replacement of hydrogen atoms with deuterium atoms in the side chains of the amino acids will not result in significant shifts of the amide I band. Hence it can be seen that there is not much difference in the position of the amide I band resulting from triple labelling. The greatest effect is observed after ¹⁵N labelling. ¹⁵N labelling is beneficial for the amide II band (1520–1550 cm⁻¹), as it is dominated by N-H bending vibration [4]. Thus, after ¹⁵N labelling, the amide II will shift to a lower frequency, which will leave the amide II region between 1520 and 1550 cm⁻¹ free from overlap so that the amide II band from the unlabelled protein can be monitored without any interference from the amide II band of the labelled protein [4].

The first study [4] to report the application of isotope-edited FTIR spectroscopy investigated the interaction between two bacterial proteins, namely HPr and IIA^mil (mannitol transporter IIA) of *Escherichia coli*. The amide I band of the labelled HPr protein was found to shift by approx. 45 cm⁻¹ towards lower frequency. An analysis of the amide I band positions of the interacting protein, as revealed using second-derivative analysis, was found to be virtually unchanged. Hence it can be concluded that the interaction between these two proteins does not lead to a major change in their protein secondary structure. Indeed, subsequent to the FTIR study, the NMR structure of the complex between the HPr and IIA^mil has been determined and this shows that, other than some subtle changes in the interface regions, where the two proteins interact, there are no global changes in the secondary and tertiary structures of the protein [6].

Isotope-edited FTIR spectroscopy has also been used to study the interaction between G-CSF and its receptor, corresponding to the extracellular Ig domain [7]. The entire extracellular domain of the G-CSF receptor consists of six distinct structural domains with each one consisting of approx. 100 amino acid residues. Those six distinct domains include the Ig-like domain. As can be seen from Figure 3 (middle trace), there is extensive overlap for the G-CSF–Ig complex when both proteins are unlabelled, making it very difficult to associate the peaks to a particular protein. However, when one of the two interacting proteins (G-CSF) is labelled, the amide I bands of the labelled protein shifts to a lower frequency, revealing the amide I band of the unlabelled Ig. The band at 1610 cm⁻¹ in the spectrum of the labelled G-CSF–Ig complex originates from the α-helical structure in G-CSF. Isotopic labelling has been used to monitor the thermal stability of two proteins that are bound to each other. The effect of increasing temperature (20 to 90 °C) on the second-derivative spectra of ¹³C,¹⁵N-labelled G-CSF–Ig complex is shown in Figure 4. At 20 °C, the major amide I band at 1610 cm⁻¹ is due to α-helix in ¹³C,¹⁵N-labelled G-CSF, and the amide I band at 1632 cm⁻¹ is due to β-sheet structure in the Ig domain. With increasing temperature, the intensity of amide I band at 1610 cm⁻¹, corresponding to helical structure in ¹³C,¹⁵N-labelled G-CSF decreases. Simultaneously, there is an increase in the amide I band at 1574 cm⁻¹. From these analyses, the thermal denaturation temperatures of both Ig and G-CSF have been obtained while they are part of a complex. It was found that the $T_m$ of the
secondary structures in Ig domain and G-CSF are different when they are bound to each other compared with their free states. Thus the $T_m$ of the β-structure in the receptor Ig domain increases by approx. 15°C and that of the helical structure in G-CSF increases by approx. 10°C in the G-CSF–receptor complex.

Isotope-edited FTIR difference spectroscopy has been used to probe the interaction between a transmembrane G-protein-coupled receptor (rhodopsin) and a peptide corresponding to a G-protein (transducin) [8]. The peptide corresponding to a segment of transducin was uniformly labelled with $^{13}$C and $^{15}$N, enabling its amide I and amide II bands to be separated from the bands of the unlabelled rhodopsin molecule. When the spectrum of the unlabelled peptide–rhodopsin complex is subtracted from the labelled peptide–rhodopsin spectrum, an isotopic shift spectrum is obtained (see Figure 5). This contains difference bands that exclusively arise from the peptide, since the peaks from the unlabelled rhodopsin molecule are cancelled out. This study revealed that the amide I band of the peptide, upon binding to rhodopsin (Meta II state), shifts from 1680 to 1644 cm$^{-1}$ in the difference spectrum. This was interpreted as a large change in the peptide structure consistent with a change from more an unordered to a more ordered structure. Using isotope-edited FTIR difference spectroscopy, the authors demonstrated the power of vibrational spectroscopy for understanding the interaction of membrane-bound receptor with its ligand [8].

Summary

There are many situations where the most powerful methods for protein structural analysis cannot be readily applied. For example, techniques such as X-ray crystallography and NMR spectroscopy do not provide the time resolution necessary for monitoring structural changes and they cannot be readily applied to peptide and protein aggregates. CD spectroscopy provides adequate time resolution, but cannot provide structural resolution at the level of individual residues in a large peptide/protein system. Other techniques such as fluorescence spectroscopy and ESR spectroscopy can be used to probe structural changes at the residue level, but they require the use of possibly perturbing probe molecules. All of these problems can be potentially overcome by using IR spectroscopy. However, like other spectroscopic methods, IR spectroscopy also has its advantages and disadvantages. It also shares common problems with other techniques, such as overlap of peaks that hinder accurate assignment of structural elements. New experimental methods such as two-dimensional IR spectroscopy [9] in conjunction with mathematical and computational techniques have been developed to unravel the overlapping components within the broad amide bands. Through the use of these methods, it is possible to obtain detailed insights into structural changes in proteins. Isotopic labelling of either individual residues or the entire protein can improve further the potential of IR spectroscopy to assess protein structure at a higher resolution. With increasing ability to label proteins using molecular biological methods, the use of isotope-edited FTIR spectroscopy for the study of protein–protein interactions is likely to be more widely used, especially in the study of membrane proteins, protein aggregates and very large protein complexes.

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References


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