variant $tRNA_i^{Met}$. To determine whether the RNA species is released as a result of the membrane acting as a diffusion barrier or as a result of previous attachment of the tRNA species to the membrane, a germinal vesicle, previously injected with gene 1 and [$\alpha$-$^{32}$P]GTP, was placed in J buffer (not containing Triton X-100) and manually stripped of its enclosing nuclear envelope as described elsewhere[13]. When the contents of the nucleus were separated from the membrane, the variant $tRNA_i^{Met}$ partitioned with the nucleoplasmic contents (Fig. 4*h*) rather than the membrane (lane *g*).

Velocity sedimentation analysis of the intranuclear contents of the manually stripped germinal vesicle revealed that the intranuclear $tRNA_i^{Met}$ species has a mobility of ~4S (data not shown). This experiment demonstrates that the species is not associated within the nucleus in a large macromolecular complex (such as the 42S particle[15]) and, even in the absence of detergent treatment, is not bound tightly to such components as the nuclear gel, the intranuclear scaffold, or chromatin (nuclear and nucleolar).

We have demonstrated here that a human variant $tRNA_i^{Met}$ containing a base substitution within the highly conserved loop 4 region is transported inefficiently from the oocyte nucleus. Although only tRNA species having mature 5′ and 3′ termini can leave the nucleus, as noted here and in an earlier study[9], our data reveal that accurate nucleolytic processing of a tRNA molecule will not guarantee nuclear escape. In addition, the trapping of the variant $tRNA_i^{Met}$ within the oocyte nucleus demonstrates that the nuclear membrane can restrict the diffusion of a mature tRNA species and provides evidence for the existence of a tRNA transport mechanism. The behaviour of the variant $tRNA_i^{Met}$ strongly suggests that the highly conserved UCGA sequence within the loop 4 region of eukaryotic tRNA has a role in the overall transport of a tRNA molecule across the nuclear membrane. Our finding that the variant is processed slowly in addition to being transported inefficiently suggests that the mechanisms of processing and transport might be functionally inter-related. Alternatively, both processing and transport may operate by entirely independent mechanisms, each recognizing common sequences (or structure) in the tRNA molecule.

Is this human variant $tRNA_i^{Met}$ a 'pseudogene' in the sense of an inactive member of a multigene family, or might it have a function? From *in vitro* studies of several RNA polymerase III transcription systems it appears that feedback inhibition by either tRNA (U. Affolter and M. Z., unpublished observations) or 5S rRNA[16,17] occurs, presumably by the binding of RNA with a component that is present in rate-limiting amounts and which is required for transcription initiation. It is unclear, however, how this mechanism might operate in the intact cell, as tRNA once processed is rapidly excluded from the nucleus, with no detectable back transport of mature tRNA from the cytoplasm[11]. We suggest that this human variant $tRNA_i^{Met}$ (either as a mature intranuclear species, or as a slowly processed precursor) might provide the intranuclear transcription aparatus with a means of sensing the output of the multigene $tRNA_i^{Met}$ family, perhaps by the mechanism suggested by the *in vitro* studies described.

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# Inelastic neutron scattering analysis of hexokinase dynamics and its modification on binding of glucose

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**The dynamical behaviour of proteins and its significance to protein function has become the subject of considerable experimental and theoretical investigation[1,2]. The existence of atomic motion about mean positions has been well established by the temperature dependence of X-ray diffraction[3] and it has long been recognized that considerable internal mobility is required in proteins to account for hydrogen exchange measurements[4]. However, only limited experimental information, mainly obtained by optical methods[5], is available on the frequencies associated with these fluctuations. Inelastic neutron scattering is a technique capable of yielding dynamical information about a system over a wide frequency range[6,7]. Recent advances in neutron spectrometers[8] and in the theoretical understanding of the dynamics of biological macromolecules have now made it both feasible and desirable to exploit this technique in the field of biophysics. To demonstrate the potential of the method, we report here data on the frequency distribution in solution of the enzyme hexokinase and its modification brought about on binding of its substrate glucose. The results lend support to the view that ligation induces a stiffening of the enzyme structure.**

Inelastic neutron scattering arises when incident neutrons gain or lose energy as a result of interaction with the internal modes of motion of a sample. Techniques to measure these energy changes are routinely used to study the dynamics of solids and liquids[6]. In the case of organic molecules the signal is dominated by the incoherent scattering by the hydrogen nuclei and can be related to the frequency spectrum of the sample[7]. However, the application of this technique to proteins in solution is hampered by the necessarily low protein concentration (at best a few per cent) which means that all measured spectra are dominated by the scattering by the buffer. Use of buffers made from $D_2O$ minimizes this background, but despite this, several previous attempts to measure the inelastic spectra of proteins in solution have suffered from insufficient statistical accuracy. This has been overcome by the time focusing time-of-flight spectrometer IN6[8] (see Fig. 1 legend) which has recently become available at the Institut Laue-Langevin, and enables measurements to be made with good counting statistics. Inelastic neutron scattering data on polycrystalline lysozyme have been reported previously[9].

We have chosen yeast hexokinase (molecular weight 50,000) for this investigation for the following reasons. Its structure is known from X-ray crystallography[10] and consists of two large domains with a deep cleft between them. A crystallographic study of an isozyme of hexokinase complexed with glucose has shown that in the complex the two domains have rotated so as

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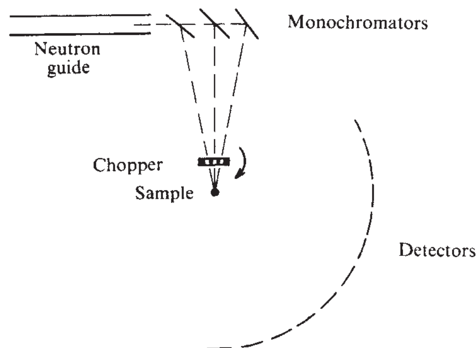


**Fig. 1** Schematic diagram of time-focusing spectrometer IN6 viewed from above. Neutrons arriving through a guide tube ($3\times20\ cm^2$) are monochromatized by a series of three pyrolytic graphite crystals with slightly different orientations. Each crystal reflects a different wavelength but all beams are focused on the sample. Pulses of neutrons are produced by a chopper which rotates about a vertical axis and is placed 40 cm before the sample. The chopper is designed so that the wavelength differences between the sub-beams are compensated by different times of chopping. In this way all elastically scattered neutrons arrive at the detector at the same time (time focusing). The instrument thus combines a high flux with a reasonable resolution ($\sim0.15$ meV or $1.2\ cm^{-1}$ at the elastic peak). In the direction perpendicular to the diagram the neutron guide tube is 20 cm in height and a geometrical focusing of the beam in that direction is achieved by curved composite monochromators. The inelastically scattered spectrum is analysed by measuring the time of flight from the sample to a bank of detectors 247 cm away. The detectors were grouped to give 24 time-of-flight spectra corresponding to scattering angles between 10 and 115°. Each time-of-flight spectrum had 512 channels. Incident neutrons of wavelength 4.1 Å (4.87 meV) were used.

to trap the glucose in the cleft[11,12]. That this conformational change on binding of glucose actually occurs in solution has been confirmed by measuring the corresponding change in the radius of gyration using small-angle X-ray scattering[13]. The interest in making inelastic neutron scattering measurements on this system follows from the speculation that the binding of glucose in the cleft and resultant conformational change may modify the vibrational spectrum of hexokinase. In particular, relative motion of the two domains may be hindered on ligation. Furthermore, it has been argued that a change in the frequency spectrum of an enzyme on ligation may be significant in the thermodynamics of enzymatic reactions[14].

The experimental strategy was to collect data successively from the buffer, the hexokinase solution and the hexokinase saturated with glucose, all at 15 °C and in the same quartz cell. Figure 2 shows corrected and scaled spectra obtained by summing counts in the range of scattering angle between 84° and 110°. The spectra of the hexokinase solution and the buffer appear similar but the statistical accuracy is sufficiently high to show that the enzyme spectrum is significantly different in form from the unsubtracted spectrum. The signal from the protein is about 15% of the total. In subtracting the buffer a factor was introduced to take into account the lower buffer content of the protein solution. We verified that variation of this scaling factor by ±5% (a wider margin of error than the true error) did not significantly affect the form of the protein spectrum (in particular the peak around $50\ cm^{-1}$). Thus, we believe that curves *c* and *d* in Fig. 2 are an accurate representation of the inelastic neutron spectra of hexokinase and hexokinase bound with glucose. Spectra were simultaneously measured at several scattering angles between 10° and 115°, and the angular dependence of the spectra can be summarized as follows. The intensity of the elastic peak of both the ligated and unligated spectra decrease roughly as $\exp(-0.89Q^2)$. The two main features of the inelastic spectrum—the peaks around 50 and $300\ cm^{-1}$—behave differently. The former increases in intensity with angle while the latter maintains the same intensity and is hardly distinguishable as a distinct peak at a scattering angle of 97°. This different behaviour with angle of high and low frequency

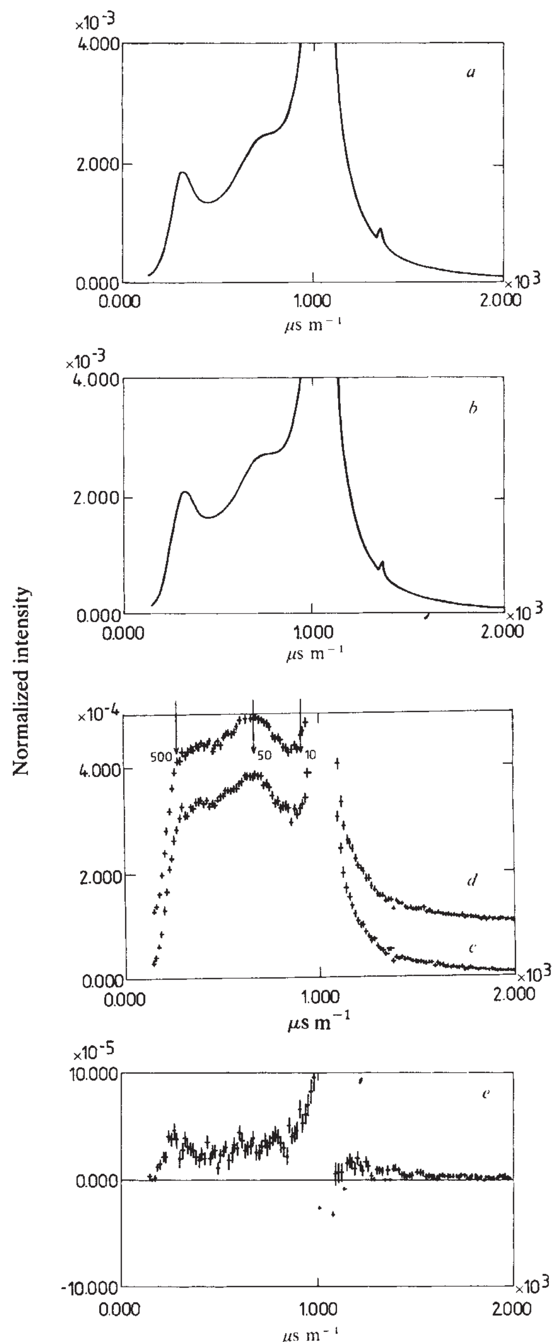


**Fig 2** Time-of-flight spectra measured at a mean angle of 97° of buffer (*a*) and hexokinase solution (*b*). The ordinates have been scaled by the elastic peaks measured with a vanadium sample. The abscissa is in time-of-flight (TOF) units ($\mu s\ m^{-1}$). Neutron energies are related to TOF by $E\ (cm^{-1}) = 42.2\times10^6/(TOF)^2$. The sharp peak on the energy loss side of both spectra is due to parasitic Bragg scattering from an aluminium window. Subtraction of the buffer (and quartz cell) scattering gives the corrected spectra of hexokinase (*c*) and hexokinase bound with glucose (*d*) (curve shifted by $10^{-4}$). Energy transfers of 10, 50 and $500\ cm^{-1}$ are indicated by arrows. The difference spectrum obtained by subtracting *c* from *d* is shown in *e*. The error bars are derived from the counting statistics and show 1 s.d. from the nominal value. Hexokinase (Sigma lot 120F–8080) in crystalline suspension was redissolved in $D_2O$ buffer containing 20 mM Tris, 0.1 M NaCl, 0.1 mM dithiothreitol, 0.1 mM sodium azide at $pD(=pH)$ 8.5 and dialysed exhaustively against the same buffer. 0.8 ml of solution (previously concentrated to $45\ mg\ ml^{-1}$) was then centrifuged at 16,000 r.p.m. for 5 min before being placed in a 1.5 mm thick quartz cell. The cell was set at 45° to the incident beam in the time-of-flight spectrometer and maintained at 15 °C. The sample chamber was maintained at a slight over pressure of helium throughout the experiment to avoid parasitic air scattering. To saturate the protein with its substrate, 1.8 mg of glucose per ml of solution was added, the glucose being in 50% (by weight) solution in the same $D_2O$ buffer. The unexchanged protons of glucose increase the number of protons in the solution by about 3%. Spectra were accumulated for about 36 h each.

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modes is consistent with the expected scattering by harmonic oscillators[7].

The dynamical modes of a protein should cover a wide range of different characteristic energies[2,5]. Some will be very similar to those found in small molecules, for example C—C bending and stretching modes and rotation of methyl groups. Characteristic energies for such local modes will be in the range 100–2,000 $cm^{-1}$. Others are collective modes specific to the secondary structure of proteins, for example longitudinal acoustic modes of helices with energies in the range 25–200 $cm^{-1}$. At still lower energies (1–50 $cm^{-1}$) 'breathing' modes as found in globular elastic bodies should occur, although the nature of these may be significantly modified by solvent damping[5]. Some of these low frequency modes may be directly linked with protein function, for example, the postulated hinge-bending mode in enzymes having a substrate binding cleft[15]. The inelastic spectra that we have measured extend up to more than 500 $cm^{-1}$, confirming the expected broad frequency spread of protein motions.

There are small but distinct differences between the spectra given by the enzyme with and without substrate bound (see Fig. 2*e*). First, there is an overall reduction of 5–10% in the intensity of inelastic scattering on glucose binding. This difference could be due *a priori* to a reduced number of protons in ligated hexokinase (resulting from a higher degree of exchange) or to a genuine modification of the enzyme dynamics. The first hypothesis is unlikely as the number of exchangeable protons is relatively small (20% of all protons) and is likely to decrease rather than increase with the tightening associated with substrate binding. Furthermore, neither the intensity nor the width of the elastic peak changes on ligation, suggesting that the total number of protons is unaltered. Thus, we conclude that there is a real modification of the dynamics of hexokinase on binding glucose. That the difference spectrum is positive suggests that the ligated enzyme has fewer and/or smaller amplitude vibrational modes, in accordance with the view that ligation induces a stiffening of the enzyme structure.

Second, Fig. 2*e* clearly shows that the shape of the difference profile differs from that of the total inelastic scattering by hexokinase. Thus, while ligation appears to affect both local and collective modes, different frequencies are affected to different extents. In particular, the low frequency modes ($<40$ $cm^{-1}$) are more markedly affected, as was also found to be the case on binding an inhibitor to lysozyme[9]. The hinge-bending mode of lysozyme has been calculated[15] to be between 5 and 15 $cm^{-1}$, and although no analogous calculations have been done for hexokinase, it is possible that the peak in the difference profile at 30 $cm^{-1}$ corresponds to the disappearance of such a mode on closing of the cleft around the glucose substrate.

Intramolecular vibrational modes contribute to the enthalpy and entropy of a protein and it has been proposed that changes in the vibrational spectrum may be important in the thermodynamics of protein reactions[14]. Our results suggest that there is a reduction in the vibrational enthalpy of hexokinase on glucose binding. However, it has been shown recently that there is practically no overall change of enthalpy on binding of glucose[16]. This is not inconsistent with our finding, as a reduction in the enthalpy of the vibrational modes could be compensating for the burying of a charged group. Indeed, we note that such a group (Asp 189) is reported to be removed from contact with the solvent as a result of the conformational change[13]. In future experiments we aim to extract from the variation with angle of the inelastic scattering spectra the frequency distribution of vibrational modes of the sample[7]. This would allow estimation of the change in the vibrational contributions to the entropy and enthalpy on ligation.

In this exploratory application of inelastic neutron scattering, we have established that it is now feasible to use measurements of the frequency spectrum of proteins in solution to characterize the change in dynamical state associated with binding a substrate molecule. Clearly, further experiments of this kind will be needed to confirm the magnitude and significance of the observed changes. Several theoretical studies of protein fluctuations have been made by molecular dynamics simulations[2,15,17,18] and normal mode analysis[19], from which it is possible to predict inelastic neutron spectra. A more detailed interpretation of the inelastic neutron scattering spectrum of hexokinase would require careful comparison with the results of such calculations, although this is not yet practicable for such a large protein. Alternatively, one can envisage the use of selective labelling (for example, protonation of certain residues in an otherwise fully deuterated protein) to aid in assignment of particular modes. Thus, our results demonstrate the potential of inelastic neutron scattering for investigating the dynamic aspects of protein function and testing the theoretical understanding of protein dynamics.

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# Errata

In the letter 'Pluripotent embryonic stem cell lines can be derived from $t^{w5}/t^{w5}$ blastocysts' by T. Magnuson *et al.*, *Nature* **298**, 750–753 (1982), in Fig. 2 the lowest bands on the gels should be labelled 17 (kilobases). In line 3 of Fig. 2 legend $129(t^0/t^{w5})$ is incorrect and should read $129(t^0/t^{w12})$.

In the letter 'Potent new inhibitors of human renin' by M. Szelke *et al.*, *Nature* **299**, 555–557 (1982), structure 4 in Table 1 erroneously contains the superscript R over the Phe-Phe peptide bond.

# Corrigenda

In the letter 'Direct imaging of travelling Rayleigh waves by stroboscopic X-ray topography' by R. W. Whatmore *et al.*, *Nature* **299**, 44–46 (1982), the references to 'half-wavelength' or '$\pi/2$' in Fig. 4 legend and in the relevant discussion in the text should read 'one wavelength' or '$\pi$'.

In the News and Views article 'Monoclonal antibodies to human malignant melanoma' by R. A. Reisfeld, *Nature* **298**, 325–326 (1982), the names of two individuals who contributed considerably to the work described were omitted: Drs K. O. Lloyd and L. J. Old of the Memorial Sloan Kettering Cancer Center, New York.

The following note relates to the letter 'Do graded units of accretionary spheroids from the Barberton Greenstone Belt indicate Archaean deep water environment?' by I. G. Stanistreet *et al.*, *Nature* **293**, 280–284 (1981). Interested readers are referred to the work of T. Heinrichs, Lithostratigraphische Untersuchungen in der Fig Tree Gruppe des Barberton Greenstone Belt zwischen Umsoli und Lomati, *Gottinger Arb. Geol. Paläont.* **22**, 119pp (1980), which was not available to the authors during the preparation of the above manuscript.

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