Protein structure change studied by hydrogen-deuterium exchange, functional labeling, and mass spectrometry

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An automated high-throughput, high-resolution deuterium exchange HPLC-MS method (DXMS) was used to extend previous hydrogen exchange studies on the position and energetic role of regulatory structure changes in hemoglobin. The results match earlier highly accurate but much more limited tritium exchange results, extend the analysis to the entire sequence of both hemoglobin subunits, and identify some energetically important changes. AllostERICALLY sensitive amide hydrogens located near amino acid resolution help to confirm the reality of local unfolding reactions and their use to evaluate resolved structure changes in terms of allosteric free energy.

Hydrogen exchange (HX) measurements can, in principle, locate protein-binding sites and structure changes and can quantify otherwise unavailable dynamic and energetic parameters (1–4). For relatively small proteins, HX can be measured at an amino acid resolved level by NMR methods. For larger, functionally more interesting proteins, other strategies are required. Earlier work (5, 6) developed a “functional-labeling” approach that can selectively label, by hydrogen-tritium (H-T) or hydrogen-deuterium (H-D) exchange, just those sites that change in any functional process. In favorable cases, the label can then be located at medium resolution by a proteolytic fragmentation method in which the fragments are quickly produced and then separated by HPLC under conditions where the loss of isotopic label is slow (6–9).

To move toward higher resolution and more comprehensive coverage of target proteins, recent work in many laboratories has coupled the HPLC separation to a second dimension of fragment resolution by online MS (10, 11). These methods tend to be labor intensive and time consuming, with limitations in throughput and comprehensiveness and in the structural resolution of functionally important changes. This article merges previous HX functional labeling and fragment separation methods with an automated MS approach termed deuterium exchange MS (DXMS) (12–18).

We are using Hb as a model system to study how protein molecules manage intramolecular signal transduction processes. Hb functions by transducing a part of the binding energy of its initially bound O₂ ligands into structure-change energy. The energy is carried through the protein to distant heme sites in the form of energetic structure changes, and there transduced back into binding energy. The initial reduced binding energy and the later enhanced binding produces the physiologically important sigmoid binding curve. In short, the currency of allosteric interactions is free energy. Trying to understand allostery without measuring free energy is like trying to understand an economic system without measuring money. A great deal of information on regulatory structure change in many proteins is now available, but mainly in a qualitative pictorial sense from “before and after” crystallographic or NMR views. How these changes participate in energy transduction and translocation has been little explored (19–22).

The HX work described here is directed toward the goal of specifying the individual allosterically important structure changes, the energetic contribution of each, and how they interact to produce the allosteric function. The methods used here provide complete protein coverage, nearly site resolution, and high-throughput efficiency. The results obtained compare well with previous highly accurate but more limited H-T exchange results and specify the position and energetic contribution of several allosterically important changes throughout the Hb molecule. We also consider the issue of concerted unfolding vs. local fluctuational HX processes and their significance for measuring the free energy of structure changes.

Methods

Hb. Human Hb was prepared from fresh red cells by standard methods and was stored frozen at –80°C. Deoxygenation used argon bubbling, dithionite, a glucose oxidase-catalase mixture, and ferrous pyrophosphate (23, 24), which also acts as a T state-selective bisphosphoglycerate analog.

Functional Labeling and Analysis. Initial isotopic labeling of Hb with tritium or deuterium was done by limited exchange-in under the conditions specified. To initiate exchange-out, Hb samples were transferred into H₂O buffer by gel filtration (Sephadex G-25 fine/0.1 M phosphate, pH 7.4/0°C/0.1 M NaCl) by using low-pressure-driven columns or centrifugal spin tubes (25).

For the fragment separation analysis, timed exchange-out samples were quenched into slow HX conditions [gel filtration, 1% trifluoroacetic acid (TFA) adjusted to pH 2.3 with NH₄OH, 0°C/50 mM GdmCl/10% glycerol to promote later sample thawing at 0°C], then were deep-frozen (~80°C) in autosampler vials pending analysis at the DXMS Proteomics Resource (Department of Medicine, University of California at San Diego). Samples were automatically thawed and analyzed by using online proteolysis with immobilized enzymes, followed by reverse phase HPLC/MS under slow HX conditions (pH 2.3, 0°C).

Loss of D-label by individual fragments during the DXMS analysis was calibrated by using Hb samples that were initially fully labeled by incubation under unfolding conditions (pD 2.3), and then moved into H₂O solution before proteolysis as was done for experimental samples. Loss corrections were made as described (Table 1).

Results shown here were obtained over a period of time by using several different MS systems. Agreement was generally excellent. The most current methodology is described.

Fragment Identification. For each digestion condition used, data were acquired by parallel MSI acquisition and data-dependent
MS2 acquisition with dynamic exclusion. The SEQUEST software program (Thermo Finnigan, San Jose, CA) identified the likely sequence of the parent peptide ions. Tentative identifications were tested with specialized DXMS data-reduction software developed in collaboration with Sierra Analytics. This software searches MS1 data for scans containing each of the peptides, selects scans with optimal signal/noise, averages the selected scans, calculates centroids, screens for peptide misidentification by comparing calculated and known centroids, then facilitates visual review of each averaged isotopic envelope, allowing an assessment of "quality" (yield, signal/noise, and resolution) and confirmation or correction (16–18).

Equipment Configuration. The equipment configuration consisted of electrically actuated high-pressure switching valves (Rhode and Yvon, Cotati, CA) connected to two position actuators (Tar Designs, Pittsburgh), except for a six-position valve-actuator multicolumn selection assembly (Valco Instruments, Houston; ref. 16). A highly modified AS3000 autosampler (Spectra-Physics), partially under external PC control, lifts the desired frozen sample from the sample well, then automatically melts and injects it under precise temperature control (13, 17, 18). The autosampler basin was further thermally insulated, and all but 20 vial positions were filled with powdered dry ice, which was sufficient to keep samples colder than −45°C for 18 h. Four Shimadzu LC-10AD HPLC pumps were operated by a Shimadzu SCL-10A pump controller. One drives the protease columns, another backflushes after sample digestion (0.05% aqueous TFA), and two deliver solvents to a downstream HPLC column for gradient elution [Solvent A: 0.05% aqueous TFA; Solvent B: 80% acetonitrile, 20% water, 0.01% TFA, pH 2.3; 1 × 50 mm C18 Vydac (Hesperia, CA)]. The valves, tubing, columns, and autosampler were contained within a refrigerator at 2.8°C, with HPLC columns immersed in melting ice.

The timing and sequence of operation of the DXMS fluids were PC controlled, running an inhouse-written LABVIEW-based program, interfaced to solid-state relays [digital input/output boards, National Instruments (Austin, TX)], controlling pumps, valve actuators, and MS data acquisition. The integrated automation of fluids allows continuous data acquisition at 30 min per sample.

DXMS Analysis. For analysis, samples were placed in the dry ice-containing sample basin of the autosampler, individually melted, then injected (45 μl) and pumped through the protease columns (0.05% TFA, 250 μl/min, 16-sec protease exposure). Proteinolysis used immobilized pepsin alone or pepsin followed by Aspergillus saitoi fungal protease type XIII [EC 3.4.23.6, Sigma; 66-μl column bed volume, coupled to 20AL support from PerSeptive Biosystems (Framingham, MA) at 30 mg/ml]. The samples were collected onto the C18 HPLC column, eluted by a linear acetonitrile gradient (5–45% B in 10 min or 30 min; 50 μl/min), and injected directly into the mass spectrometer run in either MS1 profile mode or data-dependent MS2 mode. MS analysis used Thermo Finnigan LCQ electrospray ion trap type mass spectrometer operated with capillary temperature at 200°C or an electrospray Micromass (Manchester, U.K.) Q-TOF mass spectrometer (16–18).

Stabilization-Free Energy. Amide hydrogens protected by protein molecular structure exchange slowly with solvent, at rates that are determined by the small fraction of time that the structural protection (H-bonding) is lost in some transient dynamic "opening" reaction (26, 27), as in Eq. 1. The complex steady-state expression for HX rate usually simplifies to Eq. 2 (hydroxide-catalyzed above pH ~4). The so-called EX2 region (bimolecular exchange; Eq. 2), where HX rate (kex) depends on the structural opening equilibrium constant, Kex, and pH, generally holds below pH ~10. The term kex, the intrinsic second-order HX rate constant calibrated for unprotected amides (28, 29), is applicable when amide hydrogens are freely exposed to solvent, e.g., in a concerted unfolding reaction, but perhaps not when exchange occurs by way of more constricted local fluctuations (30). When Eq. 2 is valid, the free energy of unfolding reactions that determine exchange rates, dependent on local structural stability, can be expressed in terms of measured HX rate (kex) as in Eq. 3. When some change produces a change in structural stabilization free energy, and therefore in HX rate, these relationships translate into Eq. 4 (assuming that the open state is at the same free energy in both forms).

\[
\text{closed} \overset{k_{\text{ex}}}\rightarrow \text{open} \overset{k_{\text{op}}}\rightarrow \text{exchange: } k_{\text{op}} = k_{\text{ex}}/k_{\text{op}} \quad [1]
\]

\[
k_{\text{ex}} = k_{\text{op}} k_{\text{ex}} [\text{OH}^-] = k_{\text{op}} k_{\text{ch}} \quad [2]
\]

\[
\Delta G_{\text{op}} = -RT \ln k_{\text{op}} = -RT \ln(k_{\text{ex}}/k_{\text{ch}}) \quad [3]
\]

\[
\Delta \Delta G_{\text{op}} = -RT \Delta \ln K_{\text{op}} = -RT \ln(k_{\text{ex},1}/k_{\text{ex},2}) \quad [4]
\]

Results

HX Progression. Fig. 1 recapitulates the increasing resolution of HX studies by using the Hb example. Fig. 1A shows H-T exchange results for Hb at an unresolved whole-molecule level. Hb labeled to exchange equilibrium in tritiated water was exchanged out in the oxy (R state) and deoxy (T state) forms. Some allosterically sensitive hydrogens exchange faster in oxy Hb than in deoxy Hb. In this mode, the number of sensitive hydrogens and their HX rates in either protein form cannot be determined because of the large background of unresolved sensitive and insensitive hydrogens that exchange over a wide range of time scales.

A functional labeling method illustrated in Fig. 1B focuses HX labeling on only the sites that change. The fast-exchanging oxy Hb form was partially labeled by exchange-in in tritiated water for a limited period (1 min in this case). Allosterically sensitive and insensitive sites that exchange in this time period become labeled. The Hb sample was then switched to the slow-exchanging deoxy form and moved into untritiated solvent (gel filtration, <1 min). The bound label begins to exchange-out. After some exchange-out (chase) time, label on allosterically insensitive sites (same HX rate in both forms) is largely lost, but it is retained on allosterically sensitive sites, which are slowed in deoxy Hb. Thus, the exchange-in/exchange-out sequence produces a sample with label selectively placed on functionally sensitive sites (6, 31). HX measurement then reveals the number of sensitive residues and their HX rates in both forms.

A correction for the residual background due to allosterically insensitive sites that still contribute to the HX curve (especially at early exchange-out time) can be made by doing the selective labeling in the reverse direction (exchange-in deoxy, exchange-out oxy) to obtain a curve that largely shows the residual background-insensitive hydrogens. Subtraction of the background curve yields an HX curve essentially for the sensitive sites alone (Fig. 1B Inset). Two sets of allosterically sensitive hydrogens exchange more slowly in deoxy Hb by almost 1,000-fold and 10,000-fold. Selectivity and accuracy increase with the rate difference, i.e., with the importance of the structure change, and decrease to zero as the rate difference disappears.

A fragment separation method can then identify the positions of the selectively labeled allosterically sensitive sites at medium resolution (6–9). Samples taken after different exchange-out times are plunged into slow HX conditions (pH ~2.5, 0°C) and quickly fragmented with acid protease. The fragments are separated under slow HX conditions and then analyzed for carried label. Fig. 1C shows results obtained by tritium exchange (circles) for a set of amides located on an N-terminal fragment of the α-chain (a1–29). Fig. 1D shows another set near the β-chain C terminus (on β130–146), which appears to account for
respectively, in the allosteric T to R transition (Eq. 141, 146, ref. 24). Although at the fragment level. As in Fig. 1A, the number of altered hydrogens and their rates in the two forms can then be ambiguous because of the undifferentiated background of functionally insensitive hydrogens. The functional labeling method used here overcomes this limitation by extracting only the functionally sensitive sites.

A tradeoff is that the information obtained in any given experimental set is then limited to the portion of the overall HX curve within the selective exchange-in/exchange-out time window. A survey of functional labeling options led us to adopt a protocol for initial studies that uses exchange-in for 20 min at 20°C, pH 7.4. Tritium exchange data obtained with this labeling protocol. The upper curve in Fig. 3 shows the number of amide sites labeled in the whole Hb molecule when the protein was initially exchanged in as oxy Hb (20 min, 20°C) and then exchanged-out as deoxy Hb (0°C). Tritium exchange data obtained with this protocol exhibit ~50 hydrogens per α-β dimer after 1 h of exchange-out. The DXMS results obtained by summing the fragment data described below (Fig. 4) are in good agreement. This curve is enriched in allosterically sensitive hydrogens but is still contaminated with some allosterically insensitive hydrogens.

The background curve in Fig. 3 (lower curve, deoxy-in/oxy-out), mainly shows the contribution of insensitive hydrogens. The difference between the upper curve (sensitive plus residual insensitive hydrogens) and the lower curve (largely insensitive hydrogens) gives the number of allosterically sensitive hydrogens that become measurable with this protocol. Some residual cross-contamination of the curves may exist, because of hydrogens that are only moderately sensitive (small rate ratio), or to a mismatch between exchange-in and exchange-out times, resulting in an underestimate of the number of sensitive sites. The ~30 hydrogens found account for almost half of the 75 total allosterically sensitive sites enumerated in prior tritium exchange work (33), which used variable exchange-in/exchange-out protocols to survey the entire Hb HX curve (~260 amide hydrogens).

Resolution. Fig 1 C and D compare some DXMS results to previous tritium exchange results for the fragments α1–29 and β130–146. When the same functional labeling protocol was used

The faster phase in B. The oxy/deoxy rate ratio (factors of 9-fold and 750-fold) indicate that these segments lose stabilizing interactions worth 1.2 kcal/mol and 3.6 kcal/mol in free energy, respectively, in the allosteric T to R transition (Eq. 4).

These approaches are limited. Only four fragments have so far been cleanly isolated in this way (α1–29, ref. 32; α137–141, unpublished data; β86–102, ref. 31; and β130–146, ref. 24). HPLC alone cannot resolve the large number of fragments produced by relatively nonspecific acid proteases. Even for the accessible fragments, the results do not closely specify the amino acid positions of the sensitive sites.

Coverage. As have other workers before (10, 11), we supplemented the HPLC separation with a second dimension of fragment separation by online MS (ESI MS). The trace tritium label used before was replaced by deuterium and the liquid scintillation analysis for tritium label was replaced by mass analysis. To move toward single amino acid resolution, we obtained and studied multiple overlapping subfragments using two online immobilized protease columns with differing substrate specificity in tandem (pepsin, fungal protease XIII). The 100+ fragments obtained with good yield and resolution provide redundant overlapping coverage of the entire amino acid sequence of both subunits (Fig. 2).

Published work using similar approaches has generally depended on a straightforward comparison of H-D exchange with and without the perturbation studied, analogous to Fig. 1A. Although at the fragment level. As in Fig. 1A, the number of altered hydrogens and their rates in the two forms can then be ambiguous because of the undifferentiated background of functionally insensitive hydrogens. The functional labeling method used here overcomes this limitation by extracting only the functionally sensitive sites.

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as before, good agreement was found. Also, as expected, the more intense exchange-in used in the DXMS work labeled additional allosterically sensitive sites on α1–29. It was known that the limited tritium exchange-in used before did not label the entire complement of sensitive hydrogens carried on α1–29 (ref. 32; the 20 min at 20°C exchange-in condition used is equivalent to >200 min at the 0°C condition used before).

Fig. 4 shows DXMS data for peptide fragments that span the entire length of both Hb subunits. Various HX patterns appear. A number of segments show no allosterically sensitive sites that exchange within the time window selected by the exchange-in/exchange-out protocol used. Some segments show one or two isolated sites, which may exchange by way of small structural fluctuations (30). Some other segments show larger sets of hydrogens. The hydrogens in each large set exchange at a similar rate in deoxy Hb and move in unison to a new faster rate when Hb changes from the T to the R form. This result suggests that each set is exposed to exchange by a concerted multiresidue local unfolding reaction. We focus here on the three sets of sensitive hydrogens studied before by tritium exchange.

β130–146. The β-chain C terminus, captured on the fragment β130–146, makes allosterically sensitive intrasubunit and intersubunit links that account for half of the Bohr effect (34), help

Fig. 3. The number of sites measured with the functional labeling protocol used (exchange-in 20°C, 20 min; exchange-out 0°C; upper curve in oxy/out deoxy; lower curve the reverse; pH or pDread 7.4). Filled circles show exchange-out data measured in whole Hb by established tritium methods. Comparative DXMS results (filled triangles) show the summed deuterium labeling from the fragments in Fig. 4. Equilibrium isotope corrections were applied (29).

Fig. 4. DXMS results for fragments that span the entire α-chain and β-chain (exchange-in 20 min, 20°C; exchange-out at 0°C, pH or pDread 7.4). Allosterically sensitive hydrogens in deoxy (blue) and liganded (red) Hb were obtained by subtracting the background from the raw data (both shown in gray). For liganded Hb, samples were initially exchanged out for 1 h in deoxy Hb to lose most of the background label, then switched to the liganded (R state) Hb by dilution into CO-containing buffer. The 1-h background was subtracted. Curves are drawn to fit the deoxy data, then moved onto the time axis to the oxy data to find the rate multiplication factor.
to bind the allosteric effector bisphosphoglycerate (35) and participate in interactions that cross the allosterically important $\alpha_1$–$\beta_2$ interface (36). This segment carries one of the two exceptionally sensitive sets of hydrogens seen before (Fig. 1B).

Table 1 shows data for the fragment $\beta_{130–146}$ and four subfragments. The last column in Table 1 estimates the number of allosterically sensitive sites on each fragment at zero time of the DXMS analysis. This number has been corrected for the loss of allosterically sensitive sites on each fragment at zero time of subfragments. Previous tritium exchange work found approximately seven allosterically sensitive hydrogens in this fragment, differing in rate between the T and R forms by a factor of 30 (37). The present results find approximately six allosterically sensitive sites, with a rate ratio of 25, between residues 89 (F5) and 98 (FG5).

Summary. These data associate three sets of allosterically sensitive hydrogens with known important structure changes and locate them at near amino acid resolution. Cooperative unfolding behavior is indicated, although the DXMS data do not precisely match the number of H-bonded amides in each segment. Whether this is due to methodological or data error or to other factors remains to be seen.

Discussion

The Free Energy of Structure Changes. Intramolecular signal transfer works by transducing and translocating structural free energy (see introduction). To understand these pathways, it will be necessary to connect the structure changes with the energy that they carry.

Structural free energy and changes therein can only be obtained by measuring structural equilibria. If HX rates are determined by equilibrium unfolding reactions, then the structural stabilization free energy can be obtained as in Eq. 3 with some confidence, and

Table 1. DXMS results for $\beta_{130–146}$ and subfragments

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Mass</th>
<th>Z</th>
<th>Obs</th>
<th>Theo</th>
<th>Ob</th>
<th>Bkgd</th>
<th>(Obs-bkgd)/recov</th>
<th>Zero time</th>
<th>Corrected</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\beta_{130–146}$</td>
<td>1868.01</td>
<td>1;3</td>
<td>86 (3)</td>
<td>78</td>
<td>2.6 (0.7)</td>
<td>0.0 (0.1)</td>
<td>6.0</td>
<td>4.0</td>
<td>1.25</td>
</tr>
<tr>
<td>$\beta_{140–146}$</td>
<td>838.44</td>
<td>1;2</td>
<td>62 (4)</td>
<td>78</td>
<td>2.0 (0.4)</td>
<td>0.1 (0.2)</td>
<td>2.8</td>
<td>3.5</td>
<td></td>
</tr>
<tr>
<td>$\beta_{141–146}$</td>
<td>767.41</td>
<td>1;2</td>
<td>60 (10)</td>
<td>72</td>
<td>1.7 (0.4)</td>
<td>0.1 (0.1)</td>
<td>2.4</td>
<td>3.0</td>
<td></td>
</tr>
<tr>
<td>$\beta_{130–140}$</td>
<td>1118.61</td>
<td>1</td>
<td>87 (3)</td>
<td>82</td>
<td>0.3 (0.1)</td>
<td>0.0 (0.1)</td>
<td>0.3</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>$\beta_{130–139}$</td>
<td>1047.57</td>
<td>1</td>
<td>86 (6)</td>
<td>85</td>
<td>0.3 (0.1)</td>
<td>0.1 (0.1)</td>
<td>0.3</td>
<td>0.4</td>
<td></td>
</tr>
</tbody>
</table>

Initial columns specify the following: fragment ID, mass, and charge (Z), the fraction of D-label recovered in calibrating experiments with initially fully D-labeled Hb, and the theoretically calculated recovery (refs. 28 and 29; see spreadsheet at http://hs2.med.upenn.edu/download.html). Total analysis loss time at pH 2.3, 0°C, was 11 min, including thawing and proteolysis, with a total HPLC gradient of 10 min. Subsequent columns show the following: the number of carried deuterons after 1 hr of deoxy Hb exchange-out for functionally labeled samples (obs), and for samples labeled in the reverse order to estimate the allosterically insensitive background contribution (bkgd). These data then yield the number of allosterically sensitive amide sites after 1 hr of deoxy Hb exchange-out (recov is average of losses measured and expected). The final column shows the number of sensitive sites corrected to zero exchange-out time using the observed exchange-out rate (Figs. 1 and 3). Parentheses show the total range of variation for two to five independent runs. The fragment diagram summarizes the results.


Table 2. DXMS results for $\alpha_1$-29 and subfragments (see legend to Table 1 in the main text)

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Mass</th>
<th>Z</th>
<th>Obs</th>
<th>Theo$_{30m}$</th>
<th>Obs</th>
<th>Bkgd</th>
<th>(Obs-bkgd)/recov</th>
<th>Zero time Corrected x1.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha_1$-29</td>
<td>2909.50</td>
<td>2,3</td>
<td>76(6.0)</td>
<td>70</td>
<td>5.3(0.3)</td>
<td>1.5(0.2)</td>
<td>5.2</td>
<td>6.2</td>
</tr>
<tr>
<td>$\alpha_1$-28</td>
<td>2796.42</td>
<td>2</td>
<td>74(6.0)</td>
<td>72</td>
<td>5.1(0.2)</td>
<td>1.5(0.2)</td>
<td>4.9</td>
<td>5.9</td>
</tr>
<tr>
<td>$\alpha_1$-27</td>
<td>2725.38</td>
<td>2,3</td>
<td>72(5.0)</td>
<td>72</td>
<td>5.2(0.1)</td>
<td>1.4(0.2)</td>
<td>5.3</td>
<td>6.4</td>
</tr>
<tr>
<td>$\alpha_1$-13</td>
<td>657.74</td>
<td>2</td>
<td>72(1.0)</td>
<td>68</td>
<td>3.5(0.2)</td>
<td>0.4(0.2)</td>
<td>4.4</td>
<td>5.3</td>
</tr>
<tr>
<td>$\alpha_1$-11</td>
<td>1170.66</td>
<td>1,2</td>
<td>72(3.0)</td>
<td>73</td>
<td>3.0(0.1)</td>
<td>0.5(0.2)</td>
<td>3.4</td>
<td>4.1</td>
</tr>
<tr>
<td>$\alpha_1$-9</td>
<td>943.50</td>
<td>1</td>
<td>68(3.0)</td>
<td>74</td>
<td>2.2(0.0)</td>
<td>0.3(0.0)</td>
<td>2.7</td>
<td>3.2</td>
</tr>
<tr>
<td>$\alpha_{10}$-29</td>
<td>1984.02</td>
<td>2</td>
<td>73(4.0)</td>
<td>53</td>
<td>2.0(0.1)</td>
<td>1.0(0.1)</td>
<td>1.6</td>
<td>1.9</td>
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<tr>
<td>$\alpha_{12}$-29</td>
<td>1756.85</td>
<td>2</td>
<td>72(8.0)</td>
<td>54</td>
<td>1.5(0.1)</td>
<td>0.8(0.1)</td>
<td>1.1</td>
<td>1.3</td>
</tr>
<tr>
<td>$\alpha_{24}$-29</td>
<td>622.30</td>
<td>1</td>
<td>76(4.0)</td>
<td>57</td>
<td>0.2(0.1)</td>
<td>0.1(0.2)</td>
<td>0.1</td>
<td>0.1</td>
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</tbody>
</table>
Table 3. DXMS results for β86-102 and subfragments (see legend to Table 1 in the main text)

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Mass</th>
<th>Z</th>
<th>Obs</th>
<th>TheO&lt;sub&gt;theo&lt;/sub&gt;</th>
<th>Obs</th>
<th>Bkd</th>
<th>(Obs-bkd)/recov</th>
<th>Corrected x1.1</th>
</tr>
</thead>
<tbody>
<tr>
<td>β86-102</td>
<td>1919.90</td>
<td>1;2</td>
<td>55</td>
<td>(3.0)</td>
<td>63</td>
<td>3.4</td>
<td>(0.1)</td>
<td>5.1</td>
</tr>
<tr>
<td>β89-102</td>
<td>1634.74</td>
<td>1;2</td>
<td>52</td>
<td>(4.0)</td>
<td>64</td>
<td>2.0</td>
<td>(0.1)</td>
<td>3.0</td>
</tr>
<tr>
<td>β94-102</td>
<td>1065.51</td>
<td>1;2</td>
<td>57</td>
<td>(2.0)</td>
<td>82</td>
<td>1.1</td>
<td>(0.2)</td>
<td>1.5</td>
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<tr>
<td>β92-102</td>
<td>1305.58</td>
<td>1;2</td>
<td>52</td>
<td>(1.0)</td>
<td>72</td>
<td>1.6</td>
<td>(0.1)</td>
<td>2.3</td>
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<td>β97-102</td>
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<td>52</td>
<td>(3.0)</td>
<td>86</td>
<td>0.1</td>
<td>(0.0)</td>
<td>0.0</td>
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<td>632.34</td>
<td>1</td>
<td>89</td>
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<td>78</td>
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<td>(0.3)</td>
<td>2.0</td>
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<td>67</td>
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<td>(0.1)</td>
<td>1.0</td>
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<td>83</td>
<td>0.7</td>
<td>(0.1)</td>
<td>0.7</td>
</tr>
<tr>
<td>β92-96</td>
<td>614.28</td>
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<td>55</td>
<td>(5.0)</td>
<td>65</td>
<td>1.3</td>
<td>(0.0)</td>
<td>1.9</td>
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</table>