^{15}N Isotopic Labeling and Amide Hydrogen Exchange Rates of Oxidized Iso-1-cytochrome *c*

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We present here the first description of complete, uniform isotopic labeling of a eukaryotic cytochrome *c*, iso-1-cytochrome *c* from the yeast *Saccharomyces cerevisiae*. The availability of sufficient quantities of ^{15}N-labeled protein allows the use of heteronuclear NMR methods to assign resonances, measure amide hydrogen exchange rates, and calculate relaxation parameters for both wild type and variant proteins. Uniform ^{15}N-labeling of the protein backbone yields resolvable, site-specific probes at every amino acid residue when using heteronuclear correlation NMR experiments. Since [^{1}H^{-15}N] HSQC (heteronuclear single quantum coherence) experiments can be collected in a few minutes, amide hydrogen exchange can now be measured for protons that exchange relatively quickly, such as those found in loops or other nonregular secondary structures.

Labeled protein was expressed in yeast strain C93,[1] which produces C102T,[2] the iso-1-cytochrome *c* variant for which the cysteine at position 102[3] is replaced by threonine. C93d, the diploid of strain C93, was produced by crossing C93 to strain B2111.[4] This diploid strain is prototrophic; thus, minimal media does not need to be supplemented with amino acids. C93d is grown in 5 L of minimal media in a 7-L batch fermenter, cells are harvested, and protein purified (Supporting Information).[5] The resulting protein is greater than 95% oxidized, as judged by visible spectroscopy and analysis of upfield resonances in the proton NMR spectra. High performance liquid chromatography (HPLC)−electron spray ionization (LC-ESI) mass spectroscopy confirmed that the ^{15}N-labeled protein mass was 12 858, as expected for 100% ^{15}N enrichment and correct expression. Protein grown on minimal media is trimethylated at lysine 72 and contains covalently attached heme so that it is indistinguishable from protein produced by strains grown in rich media. A [^{1}H-^{15}N] HSQC spectrum shows well-dispersed, sharp amide ^{1}H^{-15}N resonances. The proton chemical shifts correspond quite well to those previously published[6] (T.L.B.; J.S.F.; S.M.B. Unpublished data), suggesting that the protein is properly folded (Figure 1).

To examine the utility of a uniformly ^{15}N-labeled eukaryotic cytochrome *c*, we performed a consecutive series of [^{1}H-^{15}N] HSQC experiments[7] in D_2O (Supporting Information, Figure 1). Measurement of the peak volumes in these spectra allowed us to observe the amide proton exchange behavior of iso-1-cytochrome *c*, particularly of those protons that could not be observed in homonuclear NMR experiments on unlabeled protein.[8] Each [^{1}H-^{15}N] HSQC spectrum took 28.5 min to acquire, so 13 data points were collected within 6.55 h (Figure 2B), the first time point reported for unlabeled protein.[8] Exchange rates were calculated from exponential decay curves using a nonlinear least squares fitting procedure (Figure 2B). Marmorino and co-workers[8] calculated exchange rates for 34 out of the 104 amide protons in the 108-residue protein, C102T. Using ^{15}N-labeled protein, exchange rates were calculated for 49 protons that exchanged slowly enough to be observed in at least the first three spectra.

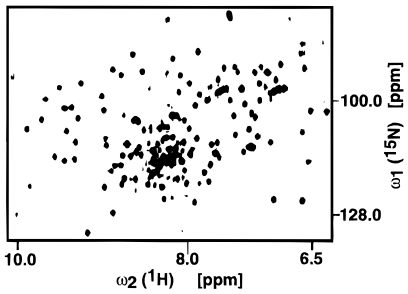

Figure 1. The [^{1}H-^{15}N HSQC] spectrum of uniformly ^{15}N labeled C102T, in 50 mM deuterated acetate in H_2O, pH 4.6, recorded at 298 K on a Bruker Avance 500 MHz spectrometer. Water suppression using WATERGATE[20] pulses was incorporated into the HSQC sequence. Frequency jumping was used to shift the transmitter to the center of the amide region of the proton spectrum. The data set consists of 128 t_1 increments, 8 scans for each, and 2 K points in t_2. The spectral width in the proton dimension was 2155 Hz; the spectral width in the nitrogen dimension was 1800 Hz. States-TPPI phase cycling[21] was used in the nitrogen dimension. Relaxation time between scans was 1.2 s. The spectrum was processed using exponential line broadening in $\omega 2$ and a shifted sinebell function in $\omega 1$.

As in previous studies, we find that amide protons in oxidized C102T exhibit typical slow, fast, and moderate exchange. This range of behavior can be observed in substructures or secondary structural elements, such as the C-terminal helix, residues 88−103 (Figure 2). We compared our rates for this complete secondary structural element with those previously determined[8] and, except for one discrepancy (discussed below), the data from the two studies correlate well (rates and protection factors are presented in Supporting Information, Table 1). The exchange rate for four protons, Glu88, Lys89, Asp90, and Asn92, could not be determined for the unlabeled protein.[8] We can now calculate exact rates of exchange for both Asp90 (k_{obs}= 0.011 ± 0.0001 min^{-1}) and Asn92 (k_{obs}= 0.00011 ± 0.0008 min^{-1}) (Supporting Information, Table 1). Notably, our calculated exchange rate for Asn92 suggests that it should have been observable in the previous study. The gains in spectral resolution due to the labeling or in signal attributable to the use of gradient-based water suppression could account for the difference between the two studies. Glu88 and Lys89 exchange fast enough that rates still could not be calculated for them.

The exchange behavior of all C-terminal helix amide protons can now be grouped into four categories: very fast (Glu88,

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(1) Mulligan-Pullyblank, P.; Spitzer, J. S.; Gilden, B. M.; Fetrow, J. S. *J. Biol. Chem.* **1996**, *271*, 8633−8645.

(2) Use of the C102T variant protein eliminates intermolecular dimerization and thus facilitates *in vitro* biophysical analysis of the protein. Its properties are virtually identical to those of true wild type iso-1-cytochrome *c*.[9,17,18,19]

(3) Iso-1-cytochrome *c* contains five extra residues at the amino terminus when aligned with other eukaryotic cytochromes *c*; thus, we follow the convention of numbering its residues from Thr(−5) to Glu103.

(4) Fetrow, J. S.; Cardillo, T. S.; Sherman, F. *Proteins: Struct., Funct., Gen.* **1989**, *6*, 372−381.

(5) The yield is approximately 0.4 mg/L; one 5-L fermentor batch is sufficient to produce one 0.5 mL NMR sample.

(6) Gao, Y.; Boyd, J.; Williams, R. J. P.; Pielak, G. J. *Biochemistry* **1990**, *29*, 6994−7003.

(7) Uniformly ^{15}N-labeled protein in aqueous buffer solution was exchanged into 50 mM deuterated acetate (pH*, uncorrected, 4.6) in D_2O, using a 10 mL Sephadex G25 spin column previously equilibrated in the same D_2O buffer. Protein concentration, determined by visible absorbance at 410 nm and an extinction coefficient of 106.1 cm^{-1} mM^{-1},[22] of the final NMR sample eluted off the G25 spin column was 0.22 mM.

(8) Marmorino, J. L.; Auld, D. S.; Betz, S. F.; Doyle, D. F.; Young, G. B.; Pielak, G. J. *Protein Sci.* **1993**, *2*, 1966−1974.

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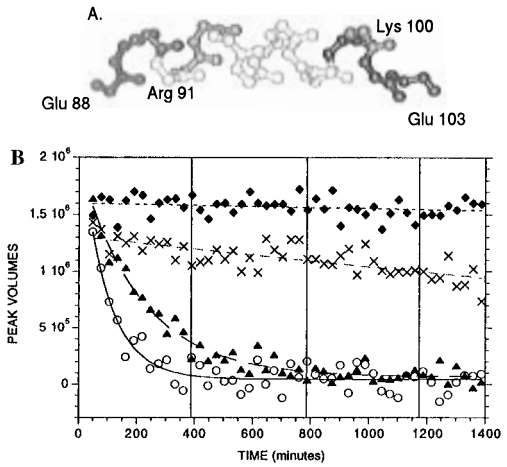

Figure 2. A. A ball-and-stick drawing of the C-terminal helix of C102T with residues containing the slowest exchanging protons colored lightest gray and residues containing the most rapidly exchanging protons colored darkest gray. The helix ends, Glu88 and Glu103, and the two residues that do not fit the pattern, Arg91 and Lys100, are specifically labeled. B. Example decay curves for fast, intermediate and slow exchanging protons in the C-terminal helix (Asp90, fast, open circles; Glu103, fast, closed triangles; Asn92, intermediate, X; Lys99, slow, closed diamonds). The dashed and dotted curves represent the best fit of the data points to a single exponential decay. Vertical lines show the time points collected during experiments done on unlabeled protein.[8]

Lys89), fast (Asp90, Lys100, Thr102, and Glu103), intermediate (Asn92, Asp93, and Ala101), and slow (Arg91, Leu94, Ile95, Thr96, Tyr97, Leu98, and Lys99) (Figure 2). With only two exceptions, these exchange rates correlate precisely with the helical nature of this secondary structural element, with the slowest exchanging protons located in the central two turns of the helix and the more rapidly exchanging protons located at the ends of the helix (Figure 2A). The two glaring exceptions to this general rule are Arg91 and Lys100, which exchange more slowly and more quickly than expected, respectively. In both cases, solvent exposure apparently plays a secondary role in determining the exchange rate. Crystallographic analysis[9] shows that the Arg91 amide forms a long hydrogen bond (3.07 Å N−O distance) to the carbonyl of Lys87, but most of the residue, except for part of the side chain guanidinium group, is completely buried in the protein interior. On the other hand, the Lys100 amide forms a short (2.83 Å) hydrogen bond to the carbonyl of Thr96, but solvent accessibility calculations[12] show that this residue is closer to the surface, as most atoms in this residue are exposed to solvent.

Interestingly, the most rapidly exchanging protons, Glu88 and Lys89, are at the N-terminus, not the C-terminus of this helix (which is the C-terminus of the protein). The fast, but measurable, exchange at the C-terminus indicates that slight helix fraying, but not significant unwinding, occurs at the protein C-terminus, corroborating site-directed spin labeling EPR studies of Cys102 in iso-1-cytochrome *c*.[10] Very rapid exchange at Glu88 and Lys89 does not mean that these residues are flexible. Backbone temperature factors from crystallographic studies[9,11] for the C-terminal helix, a structure which is not involved in crystal contacts, are slightly lower at the helix N-terminus than at the C-terminus (an average of 13.6 Å^2 for Glu88 and Lys89 compared with 15.9 Å^2 for Ala101, Thr102, and Glu103). Furthermore, solvent accessibility calculations[12] show that the amide nitrogen atom of Glu88 is exposed to a water-sized probe of 1.4 Å radius. When the probe radius is slightly decreased to 1.3 Å, the amide nitrogen of Lys 89, but no other amide nitrogens in the C-terminal helix, also becomes accessible to the probe. These observations, coupled with our results, suggest that the protein structure rigidly holds these hyperexposed amide protons toward the solvent, resulting in the very rapid exchange. This hypothesis can be tested by heteronuclear ^{15}N relaxation experiments that are now feasible with uniformly labeled protein in hand.

We can now measure exchange rates of more rapidly exchanging amide protons not previously observable, including Asp90 and Asn92 already discussed, as well as Met80 and other residues located in loops and other nonregular secondary structures. Met80 is the second heme ligand and is found in loop D, the substructure determined to be the least stable by hydrogen exchange experiments on horse cytochrome *c*.[13] Gain of the Met80 ligand is the last step in cytochrome *c* folding[14,15] and Met80 ligation is easily weakened by mutations distant from the ligation site.[16] Under native conditions, we can now observe that this proton exchanges relatively quickly with $k_{obs} = 0.018$ min^{-1} (±0.0025). Thus, rate constants for rapidly exchanging amide protons in other regions of iso-1-cytochrome *c*, besides the C-terminal helix, can now be measured for the native protein.

We have demonstrated the utility of uniformly ^{15}N-labeling iso-1-cytochrome *c* by measuring the exchange rate of protons whose exchange behavior could not be previously observed, thus allowing more complete coverage of the millisecond time scale dynamics of the protein backbone. Another important consequence of our isotopic labeling method is that we can now extend these dynamics studies to the picosecond−nanosecond time scale using heteronuclear relaxation methods. Because of the genetic malleability of the yeast system, these studies can be accomplished both on wild type protein and on variant proteins to further pursue functional, folding, and stability questions on this ideal model protein.

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Supporting Information Available: Description of media composition, table of calculated amide exchange rates and protection factors for all residues in the C-terminal helix in C1027 cytochrome *c* from *Saccharomyces cerevisiae* and figure of first [^{1}H-^{15}N] HSQC spectrum collected after exchange into D_2O with peak assignments (5 pages). See any current masthead page for ordering and Internet access instructions.

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(9) Berghuis, A. M.; Brayer, G. D. *J. Mol. Biol.* **1992**, *223*, 959−976.
(10) Qu, K.; Vaughn, J. L.; Sienkiewicz, A.; Scholes, C. P.; Fetrow, J. S. *Biochemistry* **1997**, *36*, 2884−2897.
(11) Louie, G. V.; Brayer, G. D. *J. Mol. Biol.* **1990**, *214*, 527−555.
(12) Lee, B.; Richards, F. M. *J. Mol. Biol.* **1971**, *55*, 379−400. Solvent accessibility calculations were performed using the program ACCESS, which implements the algorithm described by Lee and Richards. Standard hydrogen-added van der Waals radii and a water sized probe of radius 1.4 Å (default program parameters) were used in the calculation, except where noted in the text.
(13) Bai, Y.; Sosnick, T. R.; Mayne, L.; Englander, S. W. *Science* **1995**, *269*, 192−197.
(14) Elöve, G. A.; Bhuyan, A. K.; Roder, H. *Biochemistry* **1994**, *33*, 6925−6935.
(15) Colón, W.; Elöve, G. A.; Wakem, L. P.; Sherman, F.; Roder, H. *Biochemistry* **1996**, *35*, 5538−5549.
(16) Qin, W.; Sanishvili, R.; Plotkin, B.; Schejter, A.; Margoliash, E. *Biochim. Biophys. Acta* **1995**, *1252*, 87−94.
(17) Cutler, R. L.; Pielak, G. J.; Mauk, A. G.; Smith, M. *Protein Eng.* **1987**, *1*, 95−99.
(18) Fetrow, J. S.; Horner, S. R.; Oehrl, W.; Schaak, D. L.; Boose, T. L.; Burton, R. E. *Protein Sci.* **1997**, *6*, 195−208.
(19) Cohen, D. S.; Pielak, G. J. *Prot. Sci.* **1994**, *3*, 1253−1260.
(20) Piotto, M.; Saudek, V.; Sklenar, V. *J. Biomol. NMR* **1992**, *2*, 661−665.
(21) Marion, D.; Ikura, M.; Tschudin, R.; Bax, A. *J. Magn. Reson.* **1989**, *85*, 393−399.
(22) Margoliash, E.; Frohwirt, N. *Biochem. J.* **1959**, *71*, 570−572.