

## **Chapter 4**

Refolding Kinetics of DNS-E(C85)-cyt *c*

### **INTRODUCTION**

To determine if all parts of the *Saccharomyces cerevisiae* iso-1 cytochrome *c* (cyt *c*) fold alike, the **D-A** distance distributions during folding must be examined with label placed at different parts of the protein. We determined FET kinetics during the folding of cyt *c* with a dansyl fluorophore (DNS) placed at the loop (C85). Preliminary results suggest that unlike in DNS(C102)- cyt *c*,<sup>1</sup> the acquisition of the folded structure is retarded during DNS-E(C85)-cyt *c* refolding. Several possible explanations for the observations are suggested.

## MATERIALS AND METHODS

The yeast iso-1 cytochrome *c* overexpressed in *E. Coli* incorporates a normal lysine at position 72 instead of trimethyllysine (a result of post-translational modification in yeast<sup>2</sup>). Lysine 72 is known to bind to the heme in alkaline iso-1 ferricytochrome *c*, hence, we mutated lysine 72 to arginine. The K72R/C102S variant was constructed using pET-20b(+) plasmid (Novagen) bearing *S. cerevisiae* iso-1-cyt *c* gene (C102S variant) placed after the vector-derived PelB periplasmic leader sequence<sup>3</sup> and the following primers (Gibco):

K72Rf: 5'-GAG TAC TTG ACT AAC CCA CGT AAA TAT ATT CCTGGT AC-3'

K72Rr: 5'-GTA CCA GGA ATA TAT TTA CGT GGG TTA GTC AAG TAC TC-3'

The mutation was performed using QuickChange kit (Stratagene). The PCR product was checked on agarose gel and was used to transform XL-1 Blue strain of *E. coli*. The single colonies were taken from LB agar plates (50 µg/mL ampicillin) and used to grow 5 mL cultures (LB, 50 µg/mL ampicillin). Plasmid was isolated with QIAGEN and sequenced. The L85C/K72R/C102S mutant was prepared using pET-20b(+) plasmid bearing K72R/C102S mutation and the following primers:

L85Cf: 5'-C AAG ATG GCC TTT GGT GGG **TGT** AAG AAG GAA AAA GAC AGA AAC GAC-3'

L85Cr: 5'-GTC GTT TCT GTC TTT TTC CTT CTT **ACA** CCC ACC AAA GGC CAT CTT G-3'

H39C/K72R/C102S mutant was prepared as well using the primers:

H39Cf: 5'-G CAT GGT ATC TTT GGC AGA **TGC** TCT GGT CAA GCT GAA GGG TAT  
TCG-3'

H39Cr: 5'-CGA ATA CCC TTC AGC TTG ACC AGA **GCA** TCT GCC AAA GAT ACC  
ATG-3'

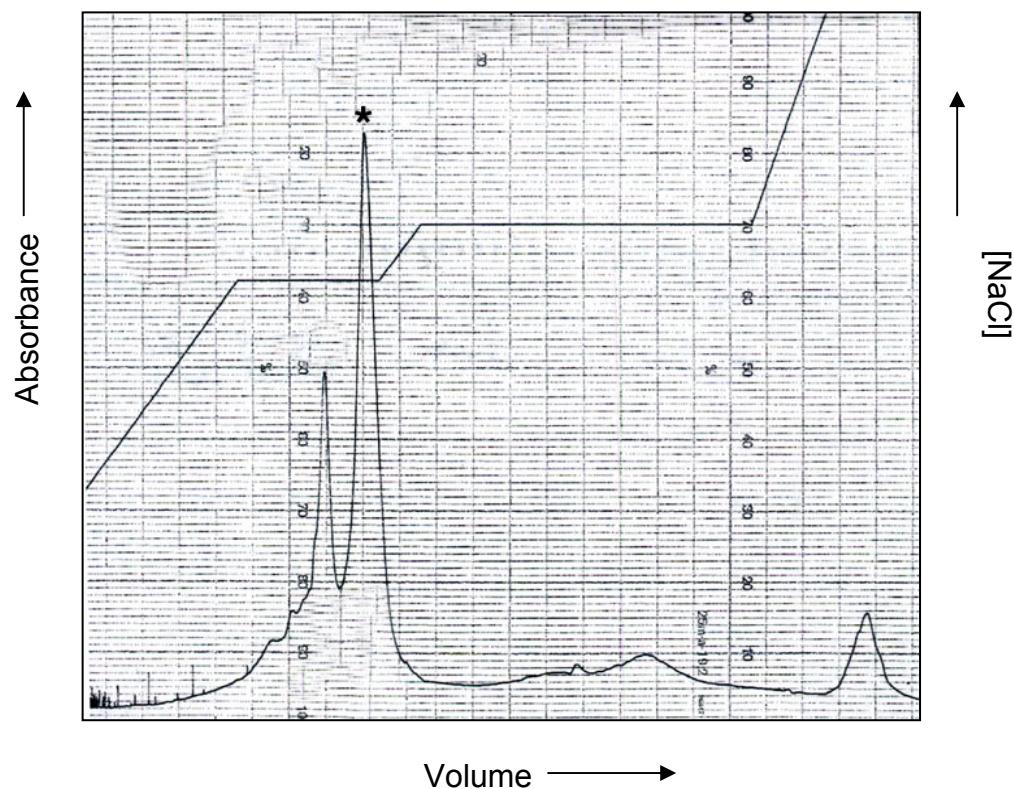
L85C/K72R/C102S (Mr = 12,669) was expressed in BL21 Star<sup>TM</sup> (DE3) *E. Coli* cells as described in Chapter 2. After CM-Sepharose purification step, the protein yield for this variant was 35 mg/L.

The protein was reduced with dithiothreitol (DTT, ICN Biomedical, Inc.) and purified by ion-exchange chromatography. The sulphydryl group of the cytochrome *c* variants was derivatized (DNS-E(C85)-cyt *c*) with thiol-reactive fluorophore 5-(((2-iodoacetyl)amino)ethyl)amino)-naphthalene-1-sulfonic acid (1,5-I-AEDANS, Molecular Probes) following protocol in Chapter 2.

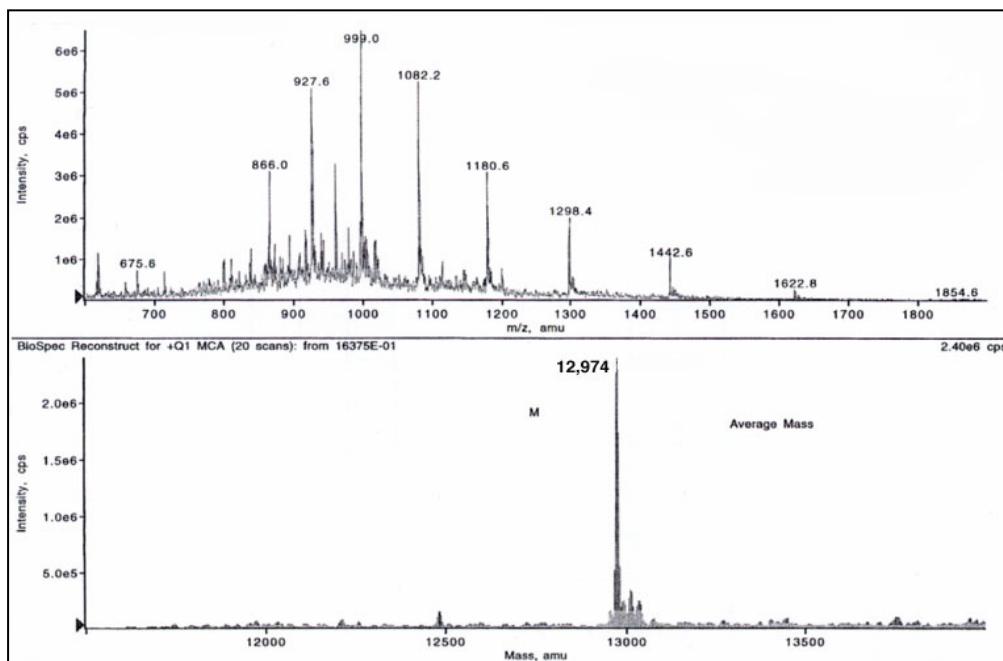
## RESULTS

### **DNS-E(C85)-cyt *c* Expression, Purification and Modification**

The FPLC ion-exchange chromatogram for separating DNS-E(C85)-cyt *c* from unlabeled protein is shown in **Figure 4.1**. The molecular weight of DNS-E(C85)-cyt *c* was 12,974.8 (Figure 4.2) as determined by mass spectral analysis. The spectroscopic properties of this variant were identical to the mutant obtained from yeast (Chapter 2).



**Figure 4.1.** Typical ion-exchange (FPLC Mono S) chromatogram for the separation of DNS-E(C85)-cyt *c*. Peak identified by \* is the labeled product.



**Figure 4.2.** Electrospray MS. DNS-E(C85)-cyt *c*. Mr = 12,974.

### DNS-E(C85)-cyt *c* refolding kinetics

Ten milliseconds after the folding is triggered, 50% of the protein ensemble has collapsed, producing a population with an average *D-A* distance of  $\sim 29$  Å (Figure 4.3). About 50 % of the protein remains in extended conformations with *D-A* distances greater than 40 Å. After 800 ms, only a slight shift of the 29 Å distribution to  $r = 27$  Å occurs. By 5 s, a small fraction of 20-Å component, a value comparable to that of the folded protein (Chapter 2) is present in the  $P(r)$  distribution. At 20 s, together, the 20-Å- and 30 Å - $r$  components account for 80% of the protein ensemble with 30-Å distribution dominant. After about 1 min, the 30-Å

distribution evolves into 20-Å distribution, however a small fraction of protein remains in this slightly more extended configuration.

## DISCUSSION

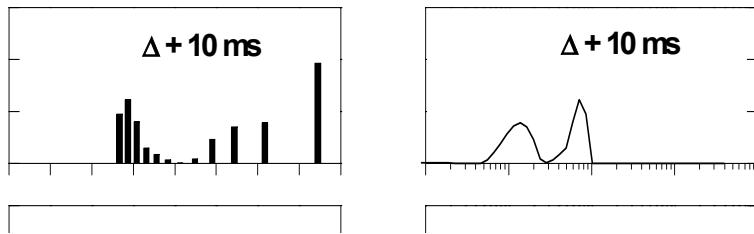
As for DNS(C102)-cyt *c*, the FET kinetics measured during DNS-E(C85)-cyt *c* folding indicate that dilution of denaturant to concentrations favoring native protein conformations ([GuHCl] = 0.13 M) does not produce a complete collapse of the polypeptide ensemble. The refolding kinetics is however slower for DNS-E(C85)-cyt *c* compared to DNS(C102)-cyt *c* and after 800 ms a large fraction (60%) of protein is in the unfolded conformation with **D-A** distance greater than 40 Å. The acquisition of the folded structure is further retarded by persistence of 30-Å component to folding times of 20 s and longer. There are several plausible explanations to the unusual behavior of FET kinetics measured during DNS-E(C85)-cyt *c* refolding.

The loop, DNS label is situated on, (the omega loop, residues 70 to 85) is the lowest energy unfolding unit.<sup>4</sup> Met 80 which is weakly axially ligated to the heme in the folded protein is located on this loop. The Met 80→Fe ligation is the first to break during unfolding and is the very last to reform during refolding of cytochrome *c*.<sup>5</sup>

On the other hand, there are two proline residues (residues 71 and 76) situated on the omega loop. Their isomerization could slow down the folding and acquisition of the folded structure as well.

The persistence of the 30-Å component could be further explained by DNS fluorophore or perhaps the mutation itself affecting Met80 → Fe ligation and/or retarding formation of the

folded polypeptide structures. Further experimental work will be required to elucidate the nature of unusual behavior of DNS-E(C85)-cyt *c* refolding kinetics.



**REFERENCES AND NOTES**

(1) Lyubovitsky, J. G.; Gray, H. B.; Winkler, J. R. *J. Am. Chem. Soc.* **2002**, *124*, 5481-5485.

(2) Pollock, W. B. R.; Rosell, F.; Twitchett, M. B.; Dumont, M. E.; Mauk, A. G. *Biochemistry* **1998**, *37*, 6124-6131.

(3) McGuirl, M. A.; Lee, J. C.; Lyubovitsky, J.; Thanyakoo, C.; Richards, J. H.; Winkler, J. R.; Gray, H. B. *BBA* **2002**, *1619*, 23-28.

(4) Bai, Y.; Sosnick, T.; Mayne, L.; Englander, S. W. *Science* **1995**, *269*, 192-197.

(5) Ridge, J. A.; Baldwin, R. L.; Labhardt, A. M. *Biochemistry* **1981**, *20*, 1662.