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Humanlike substitutions to Ω-loop D of yeast iso-1-cytochrome *c* only modestly affect dynamics and peroxidase activity

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ABSTRACT

Structural studies of yeast iso-1-cytochrome *c* (L.J. McClelland, T.-C. Mou, M.E. Jeakins-Cooley, S.R. Sprang, B.E. Bowler, Proc. Natl. Acad. Sci. U.S.A. 111 (2014) 6648–6653) show that modest movement of Ω-loop D (residues 70–85, average RMSD versus the native structure: 0.81 Å) permits loss of Met80-heme ligation creating an available coordination site to catalyze the peroxidase activity mediated by cytochrome *c* early in apoptosis. However, Ala81 and Gly83 move significantly (RMSDs of 2.18 and 1.26 Å, respectively). Ala81 and Gly83 evolve to Ile and Val, respectively, in human cytochrome *c* and peroxidase activity decreases 25-fold relative to the yeast protein at pH 7. To test the hypothesis that these residues evolved to restrict the peroxidase activity of cytochrome *c*, A81I and G83V variants of yeast iso-1-cytochrome *c* were prepared. For both variants, the apparent pK_a of the alkaline transition increases by 0.2 to 0.3 relative to the wild type (WT) protein and the rate of opening the heme crevice is slowed. The cooperativity of acid unfolding is decreased for the G83V variant. At pH 7 and 8, the catalytic rate constant, k_{cat}, for the peroxidase activity of both variants decreases relative to WT, consistent with the effects on alkaline isomerization. Below pH 7, the loss in the cooperativity of acid unfolding causes k_{cat} for peroxidase activity to increase for the G83V variant relative to WT. Neither variant decreases k_{cat} to the level of the human protein, indicating that other residues also contribute to the low peroxidase activity of human cytochrome *c*.

1. Introduction

For many years, cytochrome *c*, Cyt*c*, was believed to act solely as an electron carrier in the electron transport chain, moving electrons between two membrane bound complexes, cytochrome *c* reductase and cytochrome *c* oxidase [1]. In 1996, it was discovered that Cyt*c* is also an important signaling agent in the intrinsic pathway of apoptosis [2], being an essential component of the apoptosome, which activates caspase 9 ultimately leading to cell death [3,4]. Recent work has shown that Cyt*c* also is phosphorylated, which appears to regulate its function in both electron transport and apoptosis [5]. Furthermore, the earliest signal in apoptosis may involve peroxidation of the inner mitochondrial membrane lipid, cardiolipin (CL), when it is bound to Cyt*c* [6]. The oxidized CL is trafficked to the outer mitochondrial membrane where it facilitates release of Cyt*c* into the cytoplasm followed by binding of Cyt*c* to Apoptotic protease activating factor 1 to form the apoptosome.

To be an effective signaling switch, the intrinsic peroxidase activity of Cyt*c* must be low, to prevent adventitious oxidation of CL. Yeast, a species which lacks components of the apoptotic pathway [7], has Cyt*c* with a 20- to 30-fold higher intrinsic peroxidase activity than horse or human Cyt*c* [8–10]. This observation suggests that Cyt*c* has evolved to limit its intrinsic peroxidase activity, so that the earliest signal in the intrinsic pathway of apoptosis is a more effective on/off switch in higher eukaryotes. Several naturally occurring variants of human Cyt*c* that are linked to thrombocytopenia have been identified [11]. Two of these, Y48H and G41S, have been shown to have higher intrinsic peroxidase activity [12,13]. These Cyt*c* variants also show higher apoptotic activity [11,14,15]. However, the enhanced apoptotic activity of the G41S variant may not be related to the thrombocytopenia it induces [16].

To show increased peroxidase activity, Cyt*c* must undergo a conformational change that produces an open coordination site. In recent structural studies, we have shown that a relative modest movement of Ω-loop D (residues 70–85) is sufficient to cause loss of Met80 ligation to the heme [9]. Besides Met80, the residues at positions 81 and 83 of yeast iso-1-Cyt*c* show the largest displacement of the backbone when Met80 is expelled from the heme crevice (Fig. 1). The sequence of Ω-loop D is the most highly conserved segment of the primary structure of

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Abbreviations

iso-1-Cytc	yeast iso-1-cytochrome *c*
tmK72	trimethyllysine 72
WT	*E. coli*-expressed wild type iso-1-Cytc; Lys72 is not trimethylated
yWT	yeast-expressed wild type iso-1-Cytc; Lys72 is trimethylated
GdnHCl	guanidine hydrochloride
CD	circular dichroism
pK_{app}	apparent pK_a of the alkaline conformational transition

Cytc [17,18]. However, residue 81 evolves from Ala in yeast to Val in plants and some insects and Ile in vertebrates [19] and residue 83 evolves from Gly in yeast to Ala and Val in higher eukaryotes [17,18]. Given the lower intrinsic peroxidase activity of mammalian cytochromes *c* relative to yeast iso-1-Cytc [8–10], we proposed that in higher eukaryotes the residues at these positions may have evolved to sterically larger amino acid side chains that inhibit the dynamic motions necessary for peroxidase activity [9].

To test this hypothesis, we have substituted the residues at positions 81 and 83 of yeast iso-1-Cytc with the amino acids found at these positions in human Cytc (Fig. 1). We have evaluated the effect of the A81I and G83V substitutions on the thermodynamics and kinetics of the alkaline transition, acid unfolding, guanidine hydrochloride (GdnHCl) unfolding and the peroxidase activity of iso-1-Cytc. We find that these substitutions decrease peroxidase activity relative to wild type (WT) iso-1-Cytc at pH 7 and above and slow the dynamics of the alkaline transition, consistent with our hypothesis. However, at lower pH the peroxidase activity of the G83V variant is enhanced relative to WT iso-1-Cytc, apparently because of the decrease in the cooperativity of acid unfolding resulting from this substitution.

2. Materials and methods

2.1. Mutagenesis and protein purification

G83V and G83V-r, A81I and A81I-r mutagenesis primers (Invitrogen; see Table S1) were used to add the A81I and G83V mutations via PCR-based mutagenesis to the WT iso-1-cytochrome *c* (iso-1-Cytc) gene in the pRbs_BTR1 expression vector [20]. The pRbs_BTR1 expression vector is a derivative of the pBTR1 expression vector [21,22] with an optimized ribosomal binding sequence. It co-expresses yeast heme lyase allowing covalent attachment of heme to the CXXCH heme attachment sequence of iso-1-Cytc in the cytoplasm of *Escherichia coli*. The gene for WT iso-1-Cytc carries a mutation that produces a C102S substitution, which prevents disulfide dimerization during physical studies. It also codes for the wild type residue, Lys72. Expression in *E. coli* does not lead to trimethylation of Lys72 as occurs in the native host *Saccharomyces cerevisiae* [21]. Sequencing to confirm the A81I and G83V mutations was performed by Eurofins Genomics (Louisville, KY) or the Genomics Core Facility at the University of Montana.

The WT iso-1-Cytc and the A81I and G83V variants were expressed in BL21(DE3) *E. coli* cells carrying the corresponding pRbs_BTR1 vector [20,23,24]. Purification was carried out as previously reported [24–27]. Briefly, cells were broken using a Q700 sonicator (Qsonica, LLC), and the lysate was cleared via centrifugation. Following 50% ammonium sulfate saturation, precipitates were again cleared via centrifugation, and the supernatant was dialyzed against 12.5 mM sodium phosphate, pH 7.2, 1 mM EDTA, 2 mM β-mercaptoethanol (β-ME). Protein was then batch absorbed to CM-Sepharose Fast Flow resin pre-equilibrated to 50 mM sodium phosphate buffer, pH 7.2, 1 mM EDTA, 2 mM β-ME, and then eluted with a linear gradient of 0–0.8 M NaCl in 50 mM sodium phosphate buffer, pH 7.2, 1 mM EDTA, 2 mM β-ME. After concentration and exchange into 50 mM sodium phosphate at pH 7 by ultrafiltration, 1.5 mL aliquots of ~3 mg/mL protein were flash frozen in liquid nitrogen and stored at −80 °C. Aliquots were thawed for cation-exchange HPLC purification with an Agilent Technologies 1200 series HPLC and a Bio-Rad UNO S6 column (catalog no. 720-0023), as previously described [24]. Protein samples were concentrated by ultrafiltration and oxidized with $K_3[Fe(CN)_6]$, followed by separation of oxidized Cytc from the oxidizing agent using a G25 Sephadex column.

2.2. Global stability measurements by guanidine hydrochloride denaturation

Global stability measurements were performed using GdnHCl as a denaturant. Measurements were performed with an Applied Photophysics Chirascan circular dichroism (CD) spectrometer coupled to a Hamilton Microlab 500 Titrator at 25 °C, as previously discussed [24,28]. Briefly, the G83V variant at 4 μM or the A81I variant at 8 μM in 20 mM Tris, pH 7.5, 40 mM NaCl and ~6 M GdnHCl was titrated into protein at the same concentration in 20 mM Tris, pH 7.5, 40 mM NaCl in a 4 mm pathlength cuvette containing a stir bar. After each addition, the sample was stirred to mix, followed by data collection at 222 and 250 nm. Baseline correction was accomplished by subtracting the ellipticity at 250 nm from the ellipticity at 222 nm ($\theta_{222corr} = \theta_{222} - \theta_{250}$). Plots of $\theta_{222corr}$ versus GdnHCl concentration for A81I and G83V variants were fit to a two-state model, assuming a linear free energy relationship and a native state baseline that is independent of GdnHCl concentration using nonlinear least-squares methods (SigmaPlot v. 13; Systat Software, Inc.), as previously outlined [29]. The free energy of unfolding in the absence of denaturant, $\Delta G_u^{\circ\prime}(H_2O)$, and the *m*-value were extracted from these fits. Parameters are the average and standard deviation of a minimum of three independent trials.

2.3. Measurement of the alkaline conformational transition

A Beckman Coulter DU 800 spectrophotometer was used for pH titrations monitored at 695 nm and 22 ± 3 °C to measure the alkaline conformational transition, as previously described [30]. Briefly, a 600 μL solution of 200 μM oxidized G83V variant in 200 mM NaCl was prepared (2× G83V stock). The 2× G83V stock and Milli-Q water were mixed 1:1 to produce a solution of 100 μM oxidized G83V in 100 mM NaCl. For the A81I variant, a 600 μL solution of 400 μM oxidized A81I variant in 200 mM NaCl was prepared (2× A81I stock). The 2× A81I stock and Milli-Q water were mixed 1:1 to produce a solution of 200 μM oxidized A81I variant in 100 mM NaCl. pH titrations were carried out by adding equal volumes of either NaOH or HCl solutions of appropriate concentration and the 2× G83V or 2× A81I stock, as appropriate, to maintain a constant protein concentration throughout the titration. pH was measured with a Denver Instrument UB-10 pH/mV meter using an Accumet double junction semi-micro pH probe (Fisher Scientific Cat. No. 13-620-852). Absorbance at 750 nm was subtracted from absorbance at 695 nm to correct for baseline drift ($A_{695corr} = A_{695} - A_{750}$).

Plots of $A_{695corr}$ versus pH for the A81I and G83V variants were fit to a modified form of the Henderson−Hasselbalch equation, Eq. (1).

$$A_{695corr} = \frac{A_N + A_{Alk} \times 10^{n[pK_{app}-pH]}}{1 + 10^{n[pK_{app}-pH]}} \quad (1)$$

In Eq. (1), A_N is corrected absorbance at 695 nm for the native state with Met80 bound to the heme, A_{alk} is the corrected absorbance at 695 nm for the alkaline state with either Lys72, Lys73 or Lys79 as the alkaline state heme ligand [21], pK_{app} is the apparent pK_a of the alkaline transition, and *n* is the number of protons linked to the alkaline transition.