Chemistry and Physics of Lipids 179 (2014) 57–63

Contents lists available at ScienceDirect

Chemistry and Physics of Lipids

journal homepage: www.elsevier.com/locate/chemphyslip

Structural transformations of cytochrome *c* upon interaction with cardiolipin

Julia Muenzner[1], Ekaterina V. Pletneva*

Department of Chemistry, Dartmouth College, Hanover, NH 03755, United States

ARTICLE INFO

Article history:
Received 28 August 2013
Received in revised form 8 November 2013
Accepted 9 November 2013
Available online 16 November 2013

Keywords:
Apoptosis
Peroxidase
Protein folding
Heme

ABSTRACT

Interactions of cytochrome c (cyt *c*) with cardiolipin (CL) play a critical role in early stages of apoptosis. Upon binding to CL, cyt *c* undergoes changes in secondary and tertiary structure that lead to a dramatic increase in its peroxidase activity. Insertion of the protein into membranes, insertion of CL acyl chains into the protein interior, and extensive unfolding of cyt *c* after adsorption to the membrane have been proposed as possible modes for interaction of cyt *c* with CL. Dissociation of Met80 is accompanied by opening of the heme crevice and binding of another heme ligand. Fluorescence studies have revealed conformational heterogeneity of the lipid-bound protein ensemble with distinct polypeptide conformations that vary in the degree of protein unfolding. We correlate these recent findings to other biophysical observations and rationalize the role of experimental conditions in defining conformational properties and peroxidase activity of the cyt *c* ensemble. Latest time-resolved studies propose the trigger and the sequence of cardiolipin-induced structural transitions of cyt *c*.

1. Introduction

The heme protein cytochrome *c* (cyt *c*) is best known for its role as an electron carrier in respiration and found in all animals, aerobic microorganisms, and plants (Moore and Pettigrew, 1990). The structure of horse heart cyt *c*, by far the best characterized cyt *c* homolog, consists of five α-helices connected by Ω loops; the *N*- and *C*-terminal helices are in contact with each other (Bushnell et al., 1990). The heme is covalently attached to the polypeptide by two thioether linkages at Cys14 and Cys17 (Allen et al., 2003). Four pyrrole nitrogens as well as residues His18 and Met80 are ligands to the heme iron. These structural features are common among all mitochondrial cyt *c* proteins.

The folding and unfolding of cyt *c* in solution have been explored extensively (Winkler, 2004; Maity et al., 2005; Ensign et al., 2008; Chen et al., 2009; Yu et al., 2012). Hydrogen–deuterium amide exchange experiments have suggested that cyt *c* consists of five cooperative folding units (foldons) of varying thermodynamic stability that undergo folding and unfolding in a stepwise, sequential mechanism (Roder et al., 1988; Bai et al., 1995; Maity et al., 2004; Krishna et al., 2006). The terminal *N*- and *C*-helices are the regions of greatest stability, while the Met80-containing loop is the least stable substructure. The foldon theory has been probed with experiments and theory, and both showed consistent results (Maity et al., 2005; Weinkam et al., 2005; Krishna et al., 2006; Duncan et al., 2009). However, despite decades of active research, the mechanism of cyt *c* folding has remained controversial, with structural features of kinetic intermediates and alternative folding pathways being the main subject of discussion (Winkler, 2004).

In addition to its native structure, cyt *c* is known to adopt a variety of alternative conformations. At low pH and high salt, the protein becomes a molten globule: a compact state with largely preserved secondary but fluctuating tertiary structure (Ohgushi and Wada, 1983; Jordan et al., 1995; Pletneva et al., 2005; Nakamura et al., 2011). Ligand substitution frequently accompanies conformational changes of cyt *c*. At high pH, the weakly-bound Met80 ligand gets replaced by either Lys73 or Lys79 resulting in increased exposure of the heme group (Rosell et al., 1998; Assfalg et al., 2003; Cherney and Bowler, 2011). In urea- or guanidine hydrochloride (GuHCl)-unfolded protein at near neutral pH, Met80 is replaced by His26 or His33, while His18 remains bound to the heme (Colón et al., 1997). In addition to common denaturants (acid, urea, and GuHCl), many other additives destabilize the native structure of cyt *c*. Detergent micelles (Bertini et al., 2004; Naeem and Khan,

Abbreviations: ATP, adenosine triphosphate; CD, circular dichroism; CT, charge transfer; CL, cardiolipin; cyt *c*, cytochrome *c*; DOPG, dioleoyl phosphatidylglycerol; EPR, electron paramagnetic resonance; FRET, fluorescence resonance energy transfer; FTIR, Fourier transform infrared; GUV, giant unilamellar vesicles; GuHCl, guanidine hydrochloride; MALDI, matrix-assisted laser desorption/ionization; MCD, magnetic circular dichroism; NMR, nuclear magnetic resonance; RR, resonance Raman; SDS, sodium dodecyl sulfate; TR-FRET, time-resolved FRET.

* Corresponding author. Tel.: +1 6036460933.
E-mail address: ekaterina.pletneva@dartmouth.edu (E.V. Pletneva).
[1] Present address: Department of Pathology, Cambridge University, Cambridge CB2 1QP, United Kingdom.

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http://dx.doi.org/10.1016/j.chemphyslip.2013.11.002

2004; Bhuyan, 2010), anionic polymers (Antalík et al., 2003; Sun et al., 2012), alcohols (Naeem and Khan, 2004; Bágel'ová et al., 2008; Singh et al., 2011), lipid membranes (Pinheiro et al., 1997), fatty acids (Patriarca et al., 2009), many aliphatic anions (Ibanez and Herskovits, 1976), and even low ionic strength (Banci et al., 1998) or added ATP (Antalík and Bágel'ová, 1995; Snider et al., 2013) alter structure and/or stability of the protein.

2. Structural change dictates new function

Work by Kagan et al. (2005) has shown that upon binding to the mitochondrial glycerophospholipid cardiolipin (CL), cyt *c* is able to function as a peroxidase and promote CL oxidation, revealing a previously unknown, early step in apoptosis. This activity has been linked to the release of cyt *c* into the cytosol and subsequent caspase activation (Kagan et al., 2005). The discovery has renewed interest in studies of cyt *c* interactions with membranes (Kimelberg and Papahadjopoulos, 1971; Pong and Griffith, 1975; Brown and Wüthrich, 1977; de Kruijff and Cullis, 1980; Rietveld et al., 1983; Hildebrandt and Stockburger, 1989; Heimburg and Marsh, 1993) and stimulated new research. Disruption of the protein tertiary structure and loss of the Met80-heme coordination have suggested the role of these changes for cyt *c* new function but the exact mechanism of the polypeptide transformations remained unclear. Several excellent reviews about earlier investigations of cyt *c* and CL in apoptosis as well as their interactions exist (Pinheiro, 1994; McMillin and Dowhan, 2002; Bayir et al., 2006; Gonzalvez and Gottlieb, 2007; Kapralov et al., 2007; Ow et al., 2008; Kagan et al., 2009). Herein, we focus on the latest discoveries that have shed light on the structural features of CL-bound cyt *c* and its conversion into an apoptotic peroxidase.

The disordered nature of CL-bound cyt *c* has been a challenge to traditional structural methods but many site-specific probes have uncovered details of this elusive ensemble (Fig. 1) (Spooner and Watts, 1992; Heimburg and Marsh, 1993; Kawai et al., 2005; Basova et al., 2007; Kapralov et al., 2007; Kapetanaki et al., 2009; Bradley et al., 2011; Hüttemann et al., 2011; Balakrishnan et al., 2012; Hanske et al., 2012; Silkstone et al., 2012; Sinibaldi et al., 2013). Collectively, these studies have suggested that multiple modes of the protein-lipid interactions (Fig. 2) as well as different protein conformations and heme ligation states may be involved. Analysis of distance distributions from time-resolved FRET (TR-FRET) studies of dye-labeled cyt *c* has overcome the disadvantages of ensemble-averaging techniques and revealed conformational diversity of the CL-bound cyt *c* (Hanske et al., 2012; Hong et al., 2012). Protein structures vary in their degree of protein unfolding and their distribution is dependent on the choice of experimental conditions, including CL content and protein coverage of the membrane surface (Hong et al., 2012). Among these structures, extended conformers with broken contacts between *N*- and *C*-terminal helices likely dominate the peroxidase activity of the ensemble (Hanske et al., 2012). Fluorescence correlation experiments have suggested that compact ***C*** and extended ***E*** structures are not independent but undergo conformational exchange related to the break-up and reestablishment of interhelical contacts (Hong et al., 2012).

3. Modes of cyt *c*–CL interactions

Analyses of the effects of ionic strength and mutations suggest that cyt *c* binding to CL-containing membranes is guided by electrostatic forces (Sinibaldi et al., 2008; Abe et al., 2011; Hanske et al., 2012; Hong et al., 2012; Sinibaldi et al., 2013) but hydrophobic interactions also play a role, perhaps after the initial docking configuration is established. Three distinct sites on the cyt *c* surface have been suggested for interactions with CL: the A site, formed

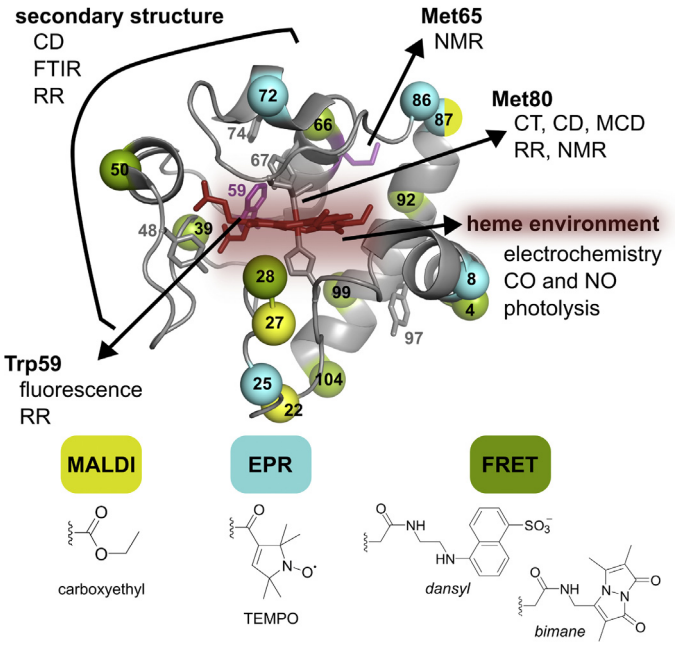

Fig. 1. Site-specific probes of the interaction of cyt *c* with CL and other anionic membranes. The heme environment (Basova et al., 2007; Kapetanaki et al., 2009; Silkstone et al., 2012), the protein secondary structure (Heimburg and Marsh, 1993; Balakrishnan et al., 2012) and single residues probing substructures of cyt *c* have been investigated with a variety of methods. Loss of Met80 ligation to the heme was illustrated by multiple spectroscopic techniques (Belikova et al., 2006; Sinibaldi et al., 2008; Bradley et al., 2011; Sinibaldi et al., 2013). The motional characteristics of ^{13}C-labeled Met65 and Met80 were probed by NMR (Spooner and Watts, 1992). The aromatic residue Trp59 was a reporter for both fluorescence (Belikova et al., 2006; Hanske et al., 2012) and RR studies (Balakrishnan et al., 2012). EPR studies and analysis of reaction products for Phe mutants explored the role of Tyr residues as radical sites (Kapralov et al., 2011; Rajagopal et al., 2013). MALDI mass spectrometry was employed to reveal sites of modification with diethylpyrocarbonate (*yellow*); these alterations prevented vesicle fusion induced by cyt *c* at low pH (Kawai et al., 2005). Labeling of Lys residues by succinimidyl-2,2,5,5-tetramethyl-3-pyrroline-1-oxyl-carboxylate (*blue*) were used to probe their proximity to the membrane by EPR (Kostrzewa et al., 2000). Modifications of installed Cys residues by small fluorescent dyes (*green*) allowed examination of conformational properties and kinetics of protein unfolding by FRET (Hanske et al., 2012; Hong et al., 2012; Muenzner et al., 2013). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

by Lys72, Lys73, Lys86 and Lys87; the C (or P) site, located near Asn52 (Rytömaa and Kinnunen, 1994, 1995); and the L site involving Lys22, Lys 27, His33, Lys25 and His26, that operates at low pH (Kawai et al., 2005). A recent mutational study ascribed a pivotal role to Lys72 and Lys79 for binding to CL (Sinibaldi et al., 2013).

Repeatedly, the region near the highly conserved residue Arg91 has been implicated in binding interactions with membranes. EPR experiments using anionic liposomes suggested that the nearby residues Lys72, Lys86 and Lys87 face the membrane surface, but argued against protein insertion into the lipid bilayer (Kostrzewa et al., 2000). Other studies indicated the protein penetration into CL-containing lipid bilayers (Gorbenko, 1999) as well as other anionic membranes (Heimburg and Marsh, 1995; Choi and Dimitriadis, 2004). Analysis of the emission maxima of dye-labeled cyt *c* variants pointed to the hydrophobic environment of the labeling site at residue 92 close to Arg91 (Hong et al., 2012; Snider et al., 2013); however, given the ease of cyt *c* dissociation, the insertion is likely not deep (Hanske et al., 2012). The interaction of the Arg guanidinum group with the phospholipid head groups is known to facilitate formation of membrane pores (Tang et al., 2007). Interestingly, an early study found that a semisynthetic cyt *c* in which Arg91 is replaced by norleucine failed to rupture membranes (Tuominen