Kinetics of Histidine Dissociation From the Heme Fe(III) in N-fragment (residues 1–56) of Cytochrome c

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We have here investigated the dissociation kinetics of the His side chains axially ligated to the heme-iron in the ferric (1–56 residues) N-fragment of horse cyt c. The ligand deligation induced by acidic pH-jump occurs as a biexponential process with different pre-exponential factors, consistent with a structural heterogeneity in solution and the presence of two differently coordinated species. In analogy with GuHCl-denatured cyt c, our data indicate the presence in solution of two ferric forms of the N-fragment characterized by bis-His coordination, as summarized in the following scheme: His18–Fe(III)–His26 = His18–Fe(III)–His33. We have found that the pre-exponential factors depend on the extent of the pH-jump. This may be correlated with the different pKa values shown by His26 and His33; due to steric factors, His26 binds to the heme–Fe(III) less strongly than His33, as recently shown by studies on denatured cyt c. Interestingly, the two lifetimes are affected by temperature but not by the extent of the pH-jump. The lower pKa for the deligation reaction required the use of an improved laser pH-jump setup, capable of inducing changes in H+ concentration as large as 1 mM after the end of the laser pulse. For the ferric N-fragment, close activation entropy values have been determined for the two histidines coordinated to the iron; this result significantly differs from that for GuHCl-denatured cyt c, where largely different values of activation entropy were calculated. This underlines the role played by the missing segment (residues 57–104) peptide chain in discriminating deligation of the “nonnative” His from the sixth coordination position of the metal.

KEY WORDS: caged proton; cytochrome c; fast kinetics; laser pH-jump; protein folding.

1. INTRODUCTION

Understanding how proteins fold is a central topic of modern structural biology and has attracted the interest of many scientists in the last years. A large body of studies has provided detailed information on protein folding and revealed that the native (i.e., the biologically active) form is reached through formation of transient intermediates (Eaton et al., 2000; Myers and Oas, 2002). Characterization of partially folded states is therefore of importance for studying molecular interactions that stabilize the polypeptide structure.

Horse cytochrome c (cyt c), a small single-chain hemoprotein of 104 residues, has been the subject of extensive studies due to its relevant role as an electron transfer protein. At present the structural...
and functional properties of the protein have been characterized in detail (Scott and Mauk, 1996). The folding process of cyt c has been investigated by a variety of methods, including ultrafast kinetic techniques (Bai, 1999; Myers and Oas, 2002; Pierce and Nall, 1997; Pierce and Nall, 2000; Yeh et al., 1997). Results obtained suggest that common mechanisms may govern protein folding pathways (Shastry et al., 1998; Yeh and Rousseau, 2000).

An interesting approach to evaluate the role played by polypeptide portions on the dynamics and the stability of the structure of globular proteins is to isolate specific chain sequences. Recently, fragments of cyt c have been obtained by partial proteolitic digestion of the protein, and their structural and functional properties investigated in detail (Di Iorio et al., 1993; Santoni et al., 2004). The heme-containing (residues 1–56) fragment of cyt c (hereafter indicated as N-fragment) contains the heme still covalently bound to a relatively large polypeptide chain, but lacks a 58-residues segment containing two important helices (i.e., the 60s and the C-terminal helices) which form with the N-terminal helix (residues 6–14) and the heme, the native hydrophobic core of the protein. The N-fragment lacks ordered secondary structure and shows bis-histidine coordination to the heme-iron; His18 is bound to the metal at the fifth coordination position as in the native conformer, His26 and/or His33 are the residues axially ligated to the iron in place of Met80 (Santucci et al., 2000). Despite the absence of ordered secondary structure, the peptide chain adopts a compact structure and shields the heme group from the solvent, as suggested by the value of the redox potential (San- toro et al., 2001). Data obtained characterize in detail (57–104 residues) fragment at neutral pH, gives rise to a complex showing native-like z-helix content and displaying redox properties close to the intact protein (Sinibaldi et al., 2001). Data obtained clearly indicate that upon complex formation the nonnative histidine is displaced by Met80 from the axial position and the native coordination to the heme-iron is restored.

In the present paper we investigate the deligation kinetics of the “nonnative” His axially ligated to the heme-Fe(III) in the N-fragment, using a laser pH-jump methodology with transient absorption detection (Abbruzzetti et al., 2001; Viappiani et al., 1998). Like GuHCl-denatured cyt c, at neutral pH the N-fragment is characterized by disordered structure and shows bis-His coordination to the heme-iron. His26 and His33 have been identified as the nonnative ligands of the metal, consistent with structural heterogeneity in solution. Our data indicate that approximately 70% of the peptide population is His18–Fe–His33 coordinated, in agreement with the fact that His33 binds to the metal more strongly than His26, as observed also for denatured cyt c. Acidification of the solution induces in the N-fragment protonation of the nonnative histidine, as it occurs in denatured cyt c.

It is well known that deligation of the “nonnative” histidine from the heme-Fe(III) at neutral pH constitutes the rate limiting step in the folding kinetics of GuHCl-denatured cyt c, this residue being a strong heme-ligand (Elöve et al., 1994; Roder et al., 1988).

The N-fragment retains a relatively large portion of the protein; thus, novel information on the deligation kinetics of the “nonnative” histidine from the metal may contribute to a deeper understanding of the role played in the process by the protein segments removed from the polypeptide chain.

The lower pKa for deligation of the nonnative histidine ligands in the N-fragment requires that the pH be lowered below at least 3.5. To this aim, we have improved the performance of the laser pH-jump setup (Abbruzzetti et al., 2001), to reach concentrations of photoreleased protons in the 10⁻³ M range. This allows to decrease the pH of the solutions well below 4 within a few nanoseconds. This improvement constitutes a major step forward, toward a general use of the laser pH-jump setup in investigations of unfolding reactions in proteins.

2. MATERIALS AND METHODS

2.1. Fragmentation of Cytochrome c

Horse heart cyt c (type VI) and thermolysin were obtained from Sigma (St. Louise, MO) and used as received. All other reagents were of analytical grade.

Digestion of horse heart cyt c to obtain the N-fragment was performed as previously described (Santucci et al., 2000). Briefly, the protein (1 mg/ml) was dissolved in 20 mM Tris–HCl buffer, pH 7.8,