Determination of the mechanism associated with the early polypeptide chain collapse is critical for the understanding of the molecular basis of protein folding. Previously, we proposed that the folding reaction of horse heart cytochrome c (cyt c) follows a biphasic mechanism. During the nascent phase of folding ($\tau < 100 \mu s$), the unfolded polypeptide chain collapses into a semicompact structure in a kinetically controlled process. It is followed by a thermodynamically controlled heme ligand exchange phase, culminating in the native state. To gain more insights into the nascent phase and its impact on the later processes, we employed TRP fluorescence and resonance Raman spectroscopies to investigate the effect of salt on the folding energy landscape of cyt c. We found that the charge screening effect of KCl accelerates the early polypeptide chain collapse and introduces heterogeneity into the subsequent folding processes.

Cyt c is a small single domain protein. It has a prosthetic heme group that is covalently attached to the polypeptide backbone through Cys14 and Cys17. In the native structure, the heme iron is axially coordinated by His18 and Met80 (the HM state). Upon denaturation, the Met80–iron bond is broken and the polypeptide chain is unfolded. The Met80 ligand can be replaced either by a solvent water molecule, leading to a water–H bond state (HW), or by His26/33, leading to a bis-His state (HH). Under acidic conditions, the His18–iron bond may also be broken, which leads to a five-coordinate state (5C) with a single water molecule as the sole axial ligand.

Cyt c has a single Trp at position 59. In the native state, the fluorescence from Trp59 is significantly quenched by the heme through Förster energy transfer because of their proximity. Because the heme is covalently linked to the polypeptide chain through Cys14 and Cys17, the Trp fluorescence intensity directly reflects the average distance between the Trp59 and the heme. Accordingly, the change in the molecular size during folding of cyt c can be estimated by Trp fluorescence measurements. In this work, the folding reaction of acid-denatured cyt c was studied with Trp fluorescence spectroscopy in the presence of various amounts of KCl (0–1.7 M) at pH 4.5. It was found that >80% of the kinetic information is lost during the deadtime of a conventional stopped-flow instrument (data not shown). To resolve the missing phase, we employed a homemade continuous-flow mixer with a deadtime of 100 µs to initiate the folding reaction. The Trp fluorescence was monitored with the previously described instrumentation from 100 µs to 20 ms.

Figure 1 shows the normalized Trp fluorescence intensity as a function of the folding time. In the absence of KCl, ~53% of the fluorescence intensity was quenched within the 100 µs nascent phase. The nascent phase amplitude increases with the KCl concentration. Remarkably, in the presence of 1.7 M KCl, the fluorescence intensity was reduced by ~95%, indicating that the polypeptide chain condensed into a structure with a native-like compactness within 100 µs. The inset in Figure 1 shows the nascent phase amplitude as a function of the KCl concentration, demonstrating that salt promotes the initial collapse of the polypeptide chain. The kinetic traces observed during the subsequent ligand-exchange phase, shown in Figure 1, were fitted with a double exponential function, as indicated by the dotted lines. The amplitude of the slow phase is small (~2–9%); hence, it will not be discussed here. The amplitude of the major phase decreases from 37 to 3% as the KCl concentration increases from 0 to 1.7 M; however, the rate constant (~1300 s$^{-1}$) is insensitive to the KCl concentration. The data suggest that the larger scale of polypeptide chain collapse induced by KCl during the nascent phase does not facilitate the rate of the ensuing reduction in molecular size. To further evaluate the impact of the initial polypeptide chain collapse on the subsequent folding processes, we followed the same reactions with resonance Raman spectroscopy. Each time-resolved Raman spectrum thus obtained was deconvoluted into a linear combination of the spectra of HM, HW, and 5C, as previously described. It is important to note that the HH intermediate is negligible under the acidic conditions applied here.

The data show that the acid-unfolded protein is comprised of 95% 5C and 5% HW. The populations of the various ligation states in the absence of KCl were plotted as a function of the folding time (Figure 2a). The time-dependent data were fitted with the following model as proposed previously, except that the side reaction from HW to HH was neglected.

$$5C \xrightarrow{k_1} HW \xrightarrow{k_2} HM \xrightarrow{k_3} 5C$$

This model, HW is an obligatory folding intermediate, 5C an off-pathway intermediate, which has to convert to HW before it can fold to the native HM state. From the best fit of the data with this model (dotted lines in Figure 2a), the rate constants $k_1$, $k_2$, and $k_3$ were determined to be 500, 1500, and 90 s$^{-1}$, respectively. Evidently, the $5C \rightarrow HW$ transition, associated with the coordination...
of His18 to the heme iron, is the slowest step for the overall folding process, despite the fact that His18 is right next to the thioether linkage between the heme and the polypeptide. On the basis of the extrapolation of the data, ~45% of HW is produced with a corresponding decrease in 5C within 100 μs.

In the presence of 1.7 M KCl, only ~10% increase in the HW population was observed at the expense of 5C within 100 μs (Figure 2b). In addition, ~15% of HM was produced, possibly due to the increased probability for Met80 to be near the heme iron owing to the early large-scale polypeptide chain collapse. The further production of HM during the subsequent ligand-exchange phase, again, can be fitted with eq 1, indicating that the basic folding mechanism is not affected by the presence of salt. Since a steady-state population of HW is present throughout the time window (the arrow indicates the 0.02 s time point), the final protein concentration for the stopped-flow measurements was 120 μM.

is quenched. We attribute the origin of the large-scale polypeptide chain collapse induced by KCl to the formation of misfolded structures in the presence of KCl. In contrast, in the subsequent ligand-exchange phase, the rate for the further reduction in the molecular size is independent of the KCl concentration. However, the overall formation rate of the native HM state is retarded by the presence of KCl. Furthermore, the presence of KCl increases the probability for the molecules to be trapped in misfolded structures with a wrong proline isomer.

Salt-induced polypeptide condensation during folding reactions has been reported in several other protein systems. Here, we show that the too-early-too-much polypeptide chain collapse induced by KCl yields some stable folding intermediates, which need to overcome a higher energy barrier to fold into their native conformation. We propose that the charge distribution on the polypeptide chain is part of the folding codon encoded in the linear amino acid sequence. The charge screening effect introduced by KCl alters the shape of the energy landscape by raising the slope of the upper rim of the funneling, thereby accelerating the early compaction of the polypeptide chain and introducing a more-rugged energy surface toward the bottom of the funnel, which increases the likelihood for the protein molecules to be trapped in misfolded structures. Previously, we proposed that the high efficiency of the folding reaction of cyt c is made possible by the rapid condensation of the polypeptide chain into a collapsed structure, and the high fidelity of the folding reaction is achieved through the subsequent thermodynamically controlled search for the global energy minimum state. The new data presented here demonstrate that a delicate balance between efficiency and fidelity is critical for the folding reaction of cyt c.

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References

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