# Probing Protein Folding with Substitution-Inert Metal Ions. Folding Kinetics of Cobalt(III)-Cytochrome *c*

F. Akif Tezcan, Jay R. Winkler,* and Harry B. Gray*

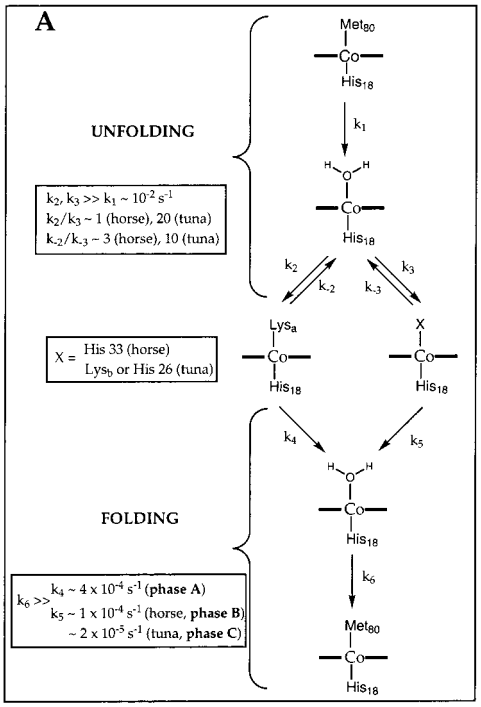
*Beckman Institute, California Institute of Technology Pasadena, California 91125*

*Received September 23, 1999*

Ligand-substitution processes at the heme strongly influence peptide backbone dynamics during the folding of cytochrome *c* (cyt *c*).[1−8] When cyt *c* is unfolded with guanidine hydrochloride (GuHCl) at pH 7, one of the axial ligands (Met 80) is replaced by a nitrogenous base from an amino acid residue; this misligation introduces an energy barrier with an associated folding time of several hundred milliseconds. A great deal of evidence points to His 26 or His 33 as the ligand in unfolded horse heart cyt *c*.[2,3,9] Nevertheless, recent studies indicate that other bases (Lys[10] or N-terminus in yeast cyt *c*[11]) can act as ligands as well. We have found that the substitution-inert heme in the Co(III) derivative of cyt *c* (Co-cyt *c*) allows a closer look at the folding kinetics and the ligands in the unfolded form of this protein.

Co-cyt *c* retains the native fold and metal ligation.[12−16] Its far-UV CD spectrum is essentially identical with that of the native protein. Unfolded Co-cyt *c* (Co-cyt $c_u$) has a far-UV CD spectrum that suggests a random-coil conformation. The Trp 59-fluorescence, fully quenched by energy transfer to the heme in the folded protein, has considerable intensity in the unfolded form, indicating loss of compact tertiary structure. Unfolding of Co-cyt *c* is accompanied by blue shifts in the absorption spectrum (Soret band, 427 to 422 nm; Q-bands, 568 to 564 and 534 to 530 nm) attributable to ligand substitution.

Folding of Co-cyt *c* was initiated by manual dilution of a concentrated denaturant solution (≥3.8 to ≤2 M GuHCl). Figure 1 summarizes folding rates and amplitudes monitored by CD spectroscopy.[17] Misligated Co-cyt $c_u$ folds much more slowly than Fe-cyt $c_u$, owing to the high activation barrier for Co(III)−ligand dissociation. Indeed, upon treating the unfolded protein with $CN^-$, folding was complete within the deadtime of manual mixing (∼5 s).[18,19] The rate constants for Co-cyt *c* folding are independent of pH between pH 5 and 7.5, consistent with a dissociative mechanism where protonation of the leaving group follows the rate-limiting step.




| B | Phase A | | Phase B | | Phase C | |
|---|---|---|---|---|---|---|
| | tn | hh | tn | hh | tn | hh |
| 5 min | 95 % | 53 % | --- | 47% | 5 % | --- |
| 10 min | 90 % | 51 % | --- | 49 % | 10 % | --- |
| 12 h | 70 % | 35 % | --- | 65 % | 30 % | --- |
| 3 days | 65 % | 25 % | --- | 75 % | 35 % | --- |

**Figure 1.** (A) Proposed ligand substitution events during Co-cyt *c* (un)-folding. (B) Amplitudes of folding phases in tuna (tn) and horse (hh) Co-cyt *c* after various incubation times for unfolding. Rate constants were determined from kinetics measured by time-resolved UV−vis ($k_1$) and CD ($k_4$, $k_5$) spectroscopy, or deduced from the amplitudes of folding phases ($k_2$, $k_{-2}$, $k_3$, $k_{-3}$) (see Supporting Information for a simulation of horse Co-cyt *c* unfolding kinetics).

The relative amplitudes of the two folding phases of Co-cyt *c* are strongly dependent on the duration of unfolding. In horse cyt *c*, the amplitude of the faster phase (phase A) decreases from 50% (after 10 min of unfolding) to 25% (after 3 days of unfolding) in favor of a slower phase (phase B) (Figure 1B). Similarly, while tuna Co-cyt *c* folding kinetics are essentially monoexponential after 10 min of unfolding, a slow phase (phase C) grows in and reaches ∼35% of the total amplitude after 3 days of unfolding. These findings indicate that ligand substitution during Co-cyt *c*

(1) Babul, J.; Stellwagen, E. *Biochemistry* **1972**, *11*, 1195−1200.
(2) Muthukrishnan, K.; Nall, B. T. *Biochemistry* **1991**, *30*, 4706−4710.
(3) Elöve, G. A.; Bhuyan, A. K.; Roder, H. *Biochemistry* **1994**, *33*, 6925−6935.
(4) Yeh, S.-R.; Rousseau, D. L. *Nat. Struct. Biol.* **1998**, *5*, 222−227.
(5) Telford, J. R.; Tezcan, F. A.; Gray, H. B.; Winkler, J. R. *Biochemistry* **1999**, *38*, 1944−1949.
(6) Telford, J. R.; Wittung-Stafshede, P.; Gray, H. B.; Winkler, J. R. *Acc. Chem. Res.* **1998**, *31*, 755−763.
(7) Shastry, M. C. R.; Sauder, J. M.; Roder, H. *Acc. Chem. Res.* **1998**, *31*, 717−725.
(8) Yeh, S.-R.; Han, S.; Rousseau, D. L. *Acc. Chem. Res.* **1998**, *31*, 727−736.
(9) A recent study (Colón, W.; Wakem, L. P.; Sherman, F.; Roder, H. *Biochemistry* **1997**, *36*, 12535−12541) has suggested His 33 as the predominant ligand.
(10) (a) Melenkivitz, R.; Russell, B. S.; Bren, K. L. Manuscript in preparation. (b) Bertini, I.; Turano, P. Manuscript in preparation.
(11) Hammack, B.; Godbole, S.; Bowler, B. E. *J. Mol. Biol.* **1998**, *275*, 719−724.
(12) Dickinson, L. C.; Chien, J. C. W. *Biochemistry* **1975**, *14*, 3526−3534.
(13) Dickinson, L. C.; Chien, J. C. W. *Biochemistry* **1975**, *14*, 3534−3541.
(14) Moore, G. R.; Williams, R. J. P.; Chien, J. C. W.; Dickinson, L. C. *J. Inorg. Biochem.* **1980**, *12*, 1−15.
(15) Co(III)-cyt *c* is synthesized by reacting metal-free cyt *c* with 10−20-fold excess $CoCl_2$ at 50 °C, pH 4.5. Metal-free cyt *c* is obtained by the treatment of cyt *c* with anhydrous HF.[12]
(16) A comparison of the stabilities of Fe(III)- and Co(III)-cyt *c* from horse, tuna, and yeast reveals that Co-cyt *c* is more stable by ∼1 kcal/mol in each species, indicating that the enhancement in stability is likely due to a stronger bond between Co(III) and S(Met 80) (see Supporting Information).
(17) The folding reaction, which was monitored by UV−visible absorption, CD, and fluorescence spectroscopy, follows biexponential kinetics. The rates and relative amplitudes agree within error for all three methods, although varying degrees of signal change are observed during the mixing deadtime.
(18) Due to the stability of the Co(III)−$CN^-$ bond, the $CN^-$ group is not displaced by S(Met 80) upon folding. This, however, does not impede the formation of the secondary and tertiary structure of cyt *c*, as evidenced by the nativelike structure of CN-Met80Ala cyt *c* (Banci, L.; Bertini, I.; Bren, K. L.; Gray, H. B.; Sompornpisut, P.; Turano, P. *Biochemistry* **1995**, *34*, 11385−11398).
(19) Cytochrome *c* folding proceeds very rapidly (on a submillisecond time scale) when imidazole is added to prevent misligation. See, for example: Shastry, M. C. R.; Roder, H. *Nat. Struct. Biol.* **1998**, *5*, 385−392.

10.1021/ja993447k CCC: $18.00 
Published on Web 12/03/1999

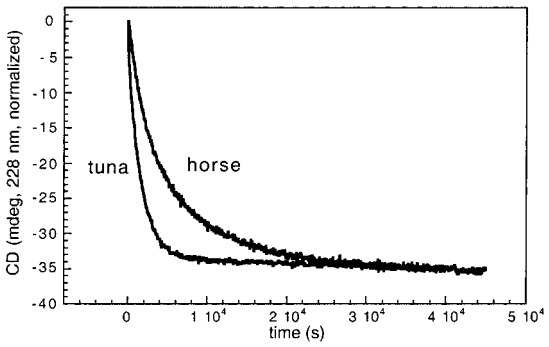



**Figure 2.** Folding kinetics of tuna and horse Co-cyt *c* (10 min of unfolding, pH 6.5, 2.0 M GuHCl).

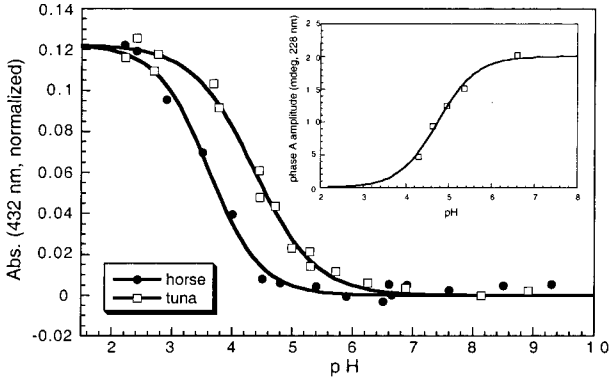



**Figure 3.** Soret absorption (432 nm) of unfolded tuna and horse Co-cyt *c* (24 h equilibration, 4.0 M GuHCl) as a function of pH. (Inset) Phase A amplitude (CD, 228 nm) of tuna Co-cyt *c* as a function of pH.

unfolding also is sluggish, enabling investigation of nonequilibrium populations of unfolded species.

We attribute the biphasic folding kinetics of Co-cyt *c* to the presence of two differently misligated unfolded protein populations. Cyanide binding to unfolded Co-cyt *c* also follows biexponential kinetics that occur on a similarly slow time scale as folding. This observation supports the view that the biphasic folding kinetics of Co-cyt *c* are not due to a complex folding mechanism but to two species that fold independently with simple exponential kinetics. The presence of multiple misligated forms has previously been suggested by pH-jump studies of unfolded cyt *c*.[9] However, parallel folding of these unfolded states has not been directly observed, probably because of the temporal proximity of Fe(III) ligand substitution to other folding processes. By separating ligand-substitution-coupled folding from backbone dynamics in the Co(III) protein, we have been able directly to detect two discrete cyt *c* folding pathways.

Comparison of tuna and horse Co-cyt *c* folding kinetics (Figures 1 and 2) suggests the probable identity of the ligands in the unfolded protein. Especially revealing is the absence of phase B in the tuna Co-cyt *c* kinetics; the composition[20] and the structure[21,22] of tuna and horse cyt *c* are essentially identical (85% sequence identity), but the tuna protein lacks His 33. Therefore, we assign phase B to the His 33-ligated population. Phase B appears to be due to the thermodynamically preferred ligand in horse Co-cyt $c_u$, in accordance with the assignment of His 33 as the predominant ligand in the unfolded iron protein.[9]

We also have examined the absorption spectra of horse and tuna Co-cyt $c_u$ as a function of pH. In both species, the pH titration reveals a dominant transition with a p$K_a$ in the range 3 to 5 (3.6 for horse; 4.4 for tuna), attributable to the (de)protonation of the axial ligand (Figure 3).[23] Furthermore, the amplitude of phase A is pH-dependent with a transition midpoint of ∼4.6 (Figure 3 inset). On the basis of the assignment of His 33 as the predominant ligand in horse Co-cyt $c_u$, we conclude that the p$K_a$ of Co(III)-bound histidine (Co(III)-His) in Co-cyt $c_u$ is ∼3.6. Consequently, we ascribe phase A to a Lys-misligated form, which should have a higher p$K_a$ than Co(III)-His.[24] It is unlikely that His 26 is sufficiently perturbed in the unfolded protein to exhibit a p$K_a$ of 4.6. It is possible that His 26 misligation is responsible for phase C in tuna Co-cyt *c* folding kinetics (Figure 1), yet the uncertainty in determining the amplitude of this phase has precluded the determination of its pH-dependence. Nevertheless, that two well-separated phases are observed during the folding of tuna Co-cyt *c* is strong evidence for Lys ligation in the unfolded protein.

Previous studies have revealed that formation of some secondary structure occurs before the ligand substitution processes that lead to Fe-cyt *c* folding.[25,26] At neutral pH and low [GuHCl], folding intermediates are trapped long enough to be studied by pulsed H/D exchange NMR.[3,27] These intermediates contain nativelike features, such as the C- and N-terminal helices that dock against each other. The ability of these helices to form despite misligation has been attributed to the large separation between His 26 or His 33 and the termini. Accordingly, when horse Co-cyt *c* is refolded at low [GuHCl] (≤0.8 M), we observe a large decrease in the far-UV CD amplitude (≤40% of total amplitude) within the deadtime of manual mixing (∼5 s). Tuna Co-cyt *c* folding, on the other hand, proceeds without the formation of such an intermediate under the same conditions. This observation can be explained by the absence of His 26 or 33 misligation in tuna Co-cyt $c_u$, and is consistent with Lys misligation. Since several Lys residues are located within or near the termini, heme ligation by any of them should prevent the formation and docking of the terminal helices.

The inertness of low-spin Co(III) played a crucial role in the development of mechanistic models for substitution reactions in octahedral metal complexes.[28,29] This property of Co(III) has allowed us to elucidate the ligand substitutions that are strongly coupled to the folding and unfolding of cyt *c*. Our approach opens new avenues for studying the folding dynamics of other proteins whose structures depend on polypeptide coordination to one or more metal centers.

**Acknowledgment.** We thank Ivano Bertini, Kara Bren, and Paola Turano for helpful discussions. This work was supported by NSF (MCB-9974477) and the Arnold and Mabel Beckman Foundation.

**Supporting Information Available:** GuHCl denaturation curves for yeast-, horse- and tuna Co-cyt *c* and relevant data; CD, fluorescence, and UV−vis spectral changes upon Co-cyt *c* unfolding; kinetics of cyanide binding to Co-cyt $c_u$; pH-dependent changes in the Soret band of tuna Co-cyt $c_u$; simulation for horse Co-cyt *c* unfolding kinetics and relevant parameters (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

JA993447K

(20) Tuna cyt *c* has 16 lysines compared to 19 in horse cyt *c*, and two histidines (His 18 and 26) compared to three in the horse protein (His 18, 26, and 33). The N-termini in both proteins are acetylated.

(21) Swanson, R.; Trus, B. L.; Mandel, N.; Mandel, G.; Kallai, O. B.; Dickerson, R. E. *J. Biol. Chem.* **1977**, *252*, 759−775.

(22) Bushnell, G. W.; Louie, G. V.; Brayer, G. D. *J. Mol. Biol.* **1990**, *214*, 585−595.

(23) Solutions for the UV−vis measurements were equilibrated for 24 h. The pH-titration fits were for a two-species equilibrium in which the species protonate independently. Nevertheless, these fits did not reveal a significant population of a second species in tuna or horse Co-cyt *c*.

(24) Similarly, pH-titration of tuna Fe(III)-cyt $c_u$ reveals a high-to-low spin transition with a midpoint (pH 6.2) significantly higher than that for horse Fe(III)-cyt $c_u$ (pH 5.2), providing additional evidence for Lys-ligation in the absence of His 33.

(25) Elöve, G. A.; Chaffotte, A. F.; Roder, H.; Goldberg, M. E. *Biochemistry* **1992**, *31*, 6876−6883.

(26) Sosnick, T. R.; Mayne, L.; Englander, S. W. *Proteins* **1996**, *24*, 413−426.

(27) Roder, H.; Elöve, G. A.; Englander, S. W. *Nature* **1988**, *335*, 700−704.

(28) Basolo, F.; Pearson, R. G. *Mechanisms of Inorganic Reactions*; John Wiley and Sons: New York, 1967.

(29) Langford, C. H.; Gray, H. B. *Ligand Substitution Processes*; W. A. Benjamin: Reading, 1966.