The significance of denaturant titrations of protein stability: a comparison of rat and baker's yeast cytochrome *c* and their site-directed asparagine-52-to-isoleucine mutants

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The residue asparagine-52 of rat cytochrome *c* and baker's yeast iso-1-cytochrome *c* was mutated to isoleucine by site-directed mutagenesis, and the unfolding of the wild-type and mutant proteins in urea or guanidinium chloride solutions was studied. Whereas the yeast mutant cytochrome unfolded in 4–7 M urea with a rate constant (*k*) of $1.7 \times 10^{-2} \text{ s}^{-1}$, the rat mutant protein unfolded with $k = 5.0 \times 10^{-2} \text{ s}^{-1}$, followed by a slow partial

refolding with $k = 5.0 \times 10^{-4} \text{ s}^{-1}$. Denaturant titrations indicated that the mutation increased the stability of the yeast cytochrome by 6.3 kJ (1.5 kcal)/mol, while it decreased that of the rat protein by 11.7 kJ (2.8 kcal)/mol. These results probably reflect structural differences between yeast iso-1 and vertebrate cytochromes *c* in the vicinity of the Asn-52 side chain.

INTRODUCTION

The side-chains of three invariant residues of tuna [1] and horse [2] cytochrome *c*, Tyr-67, Asn-52 and Thr-78, are hydrogen-bonded to the interior water molecule, W1; in yeast iso-1-cytochrome *c* (SC-iso1c) W1 is farther away from Asn-52, and only the H-bonds to Tyr-67 and Thr-78 remain present [3]. Interest in this domain of cytochrome *c* arose from the finding that mutation of Tyr-67 and Asn-52 of various cytochrome *c* species to non-H-bonding side-chains altered the stability of the protein [4–7]. Thus unfolding of recombinant SC-iso1c with the Asn-52 → Ile mutation (SC-iso1c-N52I) required larger guanidinium chloride concentrations than did the parent wild-type, SC-iso1c, due to an increase in the global stability of the protein [4,5]. Recombinant rat cytochrome *c* with the Tyr-67 → Phe mutation (RNc-Y67F) also required higher urea concentrations to reach the mid-point of denaturant titrations [6], but this was due to a drastic change in the slope of the titration curves, and extrapolation of the latter to 0 M urea indicated that the global stability of the protein had actually decreased [7].

The effects of the Tyr-67-to-Phe mutation were attributed to the hypothetical absence of W1 from the mutant protein, due to the elimination of the Tyr-67 hydroxyl group, and it was postulated that analogous consequences would result from mutations at the other side chains H-bonded to W1. Since the results obtained with SC-iso1c-N52I [4,5] contradicted this prediction, it was of interest to perform the same mutation on the rat protein.

Our experiments confirmed the observations [4,5] that the Asn-52 → Ile mutation of yeast iso-1-cytochrome *c* stabilized the folded state relative to the unfolded state in pure buffer. For the rat protein, however, the results were diametrically opposed,

and, as expected, the global stability of the molecule decreased in its Asn-52 → Ile mutant as it had with the Tyr-67 → Phe mutant [6,7]. These results show that the well-known differences in stability between yeast iso1-cytochrome *c* and its mammalian homologues [8,9] are also manifested in the different response of the proteins to the particular mutation examined here. Together with previous studies on mutations of cytochrome *c* side chains located in the immediate vicinity of the haem or closely related to it [6,7], the present results call attention to the fact that the use of midpoints of denaturant titrations as a criterion for comparing stabilities of homologous proteins can be misleading, and extrapolation to pure solvent, which is experimentally [10] and theoretically [11] justified, should be preferred.

We also report that the Asn-52 → Ile mutation of RNc and SC-iso1c had kinetic consequences on the unfolding of these cytochromes, represented by the introduction of slow steps in their chemically induced denaturation. Again, the response of the two proteins to the mutation was different, as evidenced by the appearance of a biphasic change in the unfolding of the rat cytochrome *c* mutant, but not in that of the yeast iso-1-cytochrome *c* mutant.

EXPERIMENTAL

Preparative procedures

The expression and purification of the wild-type and site-directed mutant yeast and rat cytochromes *c* were performed by the procedures described by Koshy et al. [12]. Briefly, a given cytochrome *c* gene was cloned behind the yeast iso1-cytochrome *c* gene promoter on a multicopy yeast plasmid. The yeast strain used as the expression vehicle (GM-3C-2: α , *leuc2-3*, *leu2-112*, *trp1-1*, *his4-519*, *cyc1-1*, *cyp3-1*) does not contain either of its

Abbreviations used: W1, the interior water molecule of cytochrome *c* that is hydrogen-bonded to the side chains of residues Tyr-67, Asn-52 and Thr-78; RNc, recombinant wild-type rat cytochrome *c*; SC-iso1c, recombinant wild-type baker's yeast iso-1-cytochrome *c*; RNc-N52I, RNc-Y67F and RNc-P30A, recombinant RNc carrying the Asn-52 → Ile, Tyr-67 → Phe and Pro-30 → Ala mutations respectively; SC-iso1c-N52I, recombinant SC-iso1c carrying the Asn-52 → Ile mutation.

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endogenous cytochrome *c* genes, but contains all the other yeast respiratory genes. Transformation of the yeast with cytochrome *c* genes conferred respiratory sufficiently as determined by growth on a non-fermentable carbon source. After growing large amounts of the transformed yeast, the cytochrome *c* was extracted and purified, with the last step being cation-exchange h.p.l.c. All of the recombinant cytochromes *c* separated into two chromatographic fractions in this last step. It has been shown [12] that the two fractions have the same amino acid sequence, but that the earlier-eluted fraction is acetylated at the N-terminus. The second fraction has a free N-terminal amino acid, and it is this fraction which was used in the experiments described here.

Analytical methods

Unfolding of cytochrome *c* was detected in an Aminco SLM8000 spectrofluorimeter by measuring the fluorescence emitted at 320–380 nm upon excitation at 280 nm. The large increases in the fluorescence observed upon unfolding are characteristic of cytochrome *c*, where the fluorescence of the single tryptophan residue at position 59 is strongly quenched by the haem group in the folded state [13]. Kinetic studies were carried out by monitoring the changes after manual mixing of the temperature-equilibrated solutions.

RESULTS

Kinetic studies of the unfolding of the Asn-52 → Ile mutants in the urea

The unfolding kinetics of horse cytochrome *c* in guanidinium chloride at 25 °C is a fast process, which was originally proposed to occur by a sequential mechanism [14] of the form:

where X_1 stands for an intermediate in the unfolding pathway, U represents the unfolded species, and X_2 is assumed to be the product of a dead-end pathway leading to an incorrectly folded species. Later studies [15,16] showed that a mechanism involving one folded and two unfolded species:

could also explain the kinetic observations obtained with different techniques. In eqn. (2), U_F and U_S represent fast- and slow-refolding species respectively, the latter being formed in a reaction 1000-fold slower than the major unfolding process.

The unfolding rates of RNC and SC-iso1c in urea were too fast to allow for kinetic observations using manual mixing, even when the temperature was 6 °C. However, time-dependent changes were observed upon mixing the mutant cytochromes *c* with either urea or guanidinium chloride at 25 °C, and a kinetic study was undertaken. The temperature-equilibrated solutions were manually mixed, and the fluorescence readings were recorded every 2 s, beginning 10–12 s after the initial mixing. The concentrations of protein after mixing with the denaturant varied between 5 and 10 µM. When standard high voltage was used on the photomultiplier detector, the fluorescence emitted by the wild-type rat or yeast cytochrome *c* varied between 80 and 120 arbitrary units in the absence of denaturant, and reached maximal values of 8500–9500 arbitrary units.

A representative experiment, mixing SC-iso1c-N52I at 25 °C with 5.5 M urea, is shown in Figure 1. These results could be fitted to a single first-order reaction, with a rate constant of $1.7 \times 10^{-2} \text{ s}^{-1}$. The rates and the extents of the fluorescence change increased with urea concentration, reached a maximum at 4 M urea and remained constant up to 7 M urea.

For RNC-N52I at 25 °C, the fluorescence changes upon mixing with urea were biphasic (Figure 2). The rates of the first and second reactions were very different, and could be independently estimated as $5.0 \times 10^{-2} \text{ s}^{-1}$ and $5.0 \times 10^{-4} \text{ s}^{-1}$. The final readings after the end of the second phase were 4500–4600 arbitrary units.

Titration of the unfolding equilibria

The cytochromes *c* (5 µM in 30 mM Mops buffer, pH 7.0) were mixed with solutions of 0–10 M urea or 0–8 M guanidinium chloride in the same buffer. The concentrations of the denaturant solutions were measured refractometrically, by using the empirical equations of Pace [17]. Because of the observed kinetic changes, the fluorescence of these solutions was recorded after the readings had reached stability, and checked after a further 1 h. The titrations were analysed by non-linear fitting of the data to the equation:

$$-RT \ln[(F - F_0)/(F_\infty - F)] = \Delta G_{D,aq} - m[\text{urea}] \quad (3)$$

This equation [7] results from interpreting the effect of urea as a displacement of the two-state equilibrium between the native and unfolded forms of the protein [18–20]. F_0 , F and F_∞ are the

Figure 1 Kinetics of effect of urea on the N52I mutant of yeast iso-1 cytochrome *c*

Measurements were in 5.5 M urea/30 mM Mops buffer, pH 7.0, at 25 °C. The fluorescence emission at 350 nm upon excitation at 280 nm was determined. The first reading of the fluorescence was obtained at 10 s after the initial mixing.

Figure 2 Kinetics of the effect of urea on the N52I mutant of rat cytochrome *c*

Measurements were in 8.9 M urea/30 mM Mops buffer, pH 7.0, at 25 °C. The fluorescence emission at 350 nm (excitation wavelength 280 nm) of RNC-N52I is shown. The first reading of the fluorescence was obtained at 10 s after the initial mixing.

Table 1 Parameters for the unfolding equilibrium of various cytochromes *c* and their artificial mutants, in urea solutions (unless otherwise stated): 30 mM Mops buffer, pH 7.0, 25 °C

Cytochrome	Reference	$\Delta G_{D,aq}$		m		[Denaturant] _{1/2} (M)
		(kJ/mol)	(kcal/mol)	(kJ · l · mol ⁻²)	(kcal · l · mol ⁻²)	
RNc	[6]	38.2	9.15	5.1	1.21	7.60
RNc-N52I	This work	26.6	6.36	3.1	0.74	8.13
RNc-P30A	[7]	15.7	3.76	2.9	0.70	5.38
RNc-Y67F	[6]	30.1	7.19	3.6	0.86	8.33
RNc-P30A/Y67F	[7]	28.2	6.74	4.9	1.16	5.81
SC-iso1c	This work	6.1	1.46	1.8	0.43	3.40
SC-iso1c-N52I	This work	12.1	2.90	2.8	0.66	4.40
SC-iso1c (guanidinium chloride)	This work	6.6	1.57	3.8	0.91	1.72
SC-iso1c-N52I (guanidinium chloride)	This work	12.6	3.02	5.8	1.39	2.19

fluorescence in buffer, at finite concentrations of urea and at saturation of the effect respectively; $\Delta G_{D,aq}$ is the free energy of unfolding in pure buffer; and m is the slope of the straight line obtained in this type of titrations [17].

The values of $\Delta G_{D,aq}$, m , and the mid-point concentrations of denaturant, are listed in Table 1 for RNc-N52I, SC-iso1c-N52I, their wild-type parent proteins, and several other mutant cytochromes *c*. The Asn-52 → Ile mutation had the same effect on the rat protein as the Tyr-67 → Phe mutation [6,7]: a lower $\Delta G_{D,aq}$, indicating destabilization of the folded state, but a higher [urea]_{1/2} due to a decrease in m . SC-iso1c-N52I was more stable than SC-iso1c, as previously reported [5], and m was larger.

DISCUSSION

In studies of the kinetics of unfolding and refolding of horse cytochrome *c* induced by guanidinium chloride [14–16], the results were explained by postulating the sequential mechanisms represented by eqns. (1) and (2). The rates of these reactions were in the millisecond range, their observation requiring rapid-mixing techniques [14–16]. A slower relaxation of 4 s⁻¹ accounted for only 3.3 % of the total change [16].

Our experiments showed unexpectedly slow unfolding rates of the Asn-52 → Ile mutant cytochromes *c* upon mixing with urea. SC-iso1c-N52I unfolded with a first-order rate constant of 1.7×10^{-2} s⁻¹ at 25 °C. Though this result is not strictly comparable with studies of the protein of a different species, and using a different denaturant, it is clear that the unfolding rate was affected by the mutation, since kinetic changes in this time range could not be observed when mixing the wild-type protein with either 6 M urea or 4 M guanidinium chloride at temperatures as low as 6 °C. The results were even more striking for RNc-N52I, since they revealed two kinetic phases, one with a rate constant close to that measured for SC-iso1c-N52I, followed by a slower phase that required more than 2 h to reach the final unfolded state.

The unexpected slowness of the unfolding reactions suggests that the Asn-52 → Ile mutation increases the activation-energy barriers for the unfolding path of the wild-type protein. If this straightforward explanation holds, then it is plausible that the second phase observed for the rat protein represents the secondary unfolding, $U_F \rightarrow U_S$, described by eqn. (2). The robust fluorescence emission observed at the end of the unfolding process rules out a major refolding of the protein, but it is possible that a partial refolding occurs during the second phase. Notably, although the unfolding rate of SC-iso1c-N52I is very close to that of the first phase in the unfolding of RNc-N52I, no

further changes are observed in the yeast protein mutant after the initial unfolding is completed.

With regard to the unfolding equilibria, an earlier study [7] showed that in denaturant titrations of wild-type and mutant cytochromes *c* the experimentally observed mid-points depend on both $\Delta G_{D,aq}$ and m . Table 1 demonstrates that all these possibilities can be realized in practice. Thus, for rat cytochrome *c*, $\Delta G_{D,aq}$ was decreased by three single mutations, Asn-52 → Ile, Pro-30 → Ala and Tyr-67 → Phe, and by the double mutation Pro-30 → Ala/Tyr-67 → Phe. Nevertheless, [urea]_{1/2} was higher for two of the mutants and lower for the other two, depending on the effects that the mutations had on m . Clearly, [urea]_{1/2} is not a good measure for comparing the stabilities of homologous proteins. Although the alternative possibility of analysing denaturant titrations by the procedure of extrapolation of the straight lines described by eqn. (3) has been rightly criticized [18], the handling of the data can be improved by directly fitting them to eqn. (3) with appropriate non-linear algorithms that do not include assumptions about the value of the physical property being examined. Furthermore, the extrapolation procedures were recently justified by a statistical thermodynamic treatment [11], and for cytochrome *c*, supported by comparisons between urea and guanidinium chloride titrations [7]. Furthermore, in the particular case of thioredoxin, titrations continued for almost the entire range of denaturant concentrations providing an empirical demonstration of the validity of the extrapolation procedure [10].

Accordingly, eqn. (3) and the heteropolymer theory [11], which predicts quantitatively the relations between $\Delta G_{D,aq}$ and m obtained in titrations of cytochrome *c* with urea and guanidinium chloride [7], are used in the present investigation. Following previous thermodynamic formulations [19,20], this theory interprets m as reflecting the increase in exposure of hydrophobic groups to the aqueous solvent upon unfolding. A modelling study showed that decreases in m of 30 %, although possible, are highly improbable; and in an experimental study of 154 single mutants of staphylococcal nuclease, only 10 % of the mutants had m values significantly lower than that of the wild-type protein [21]. In this regard, the behaviour of various cytochrome *c* mutants is indeed striking: in all the mutants of the rat protein at sites H-bonded to W1, and in the mutants at the Pro-30 site [22], the value of m was drastically decreased, by 30–40 %. These large changes in m are clearly attributable to the fact that all the mutated sites are major determinants of the thermodynamic stability of the native cytochrome *c* conformation, as this series of experiments was originally designed to demonstrate [6,7,22]. Thus Pro-30 [22] is part of the very rigid 27–30 sequence [23] that

contains an unusually hydrogen-bonded γ bend, and has minimal solvent accessibility [24]; Tyr-67 and Asn-52 are both hydrogen-bonded to W1, and the former is presumably similarly bonded to the iron-ligand atom, the Met-80 sulphur [1].

The most straightforward explanation of the lowered values of m is that mutations of Tyr-67 and Asn-52, on the one hand, and Pro-30, on the other, increase the solvent accessibility of the left or right interior domains of the protein, respectively. Thus the initial exposure of interior domains of the protein is larger in all these mutants than in the wild-type, and the relative change in the total amount of exposed hydrophobic groups in the presence of denaturant should be correspondingly smaller [22]. Although it must be stressed that m depends on both the initial and the final states of the unfolding equilibrium [10,25], and it is quite possible that the U states of the mutant proteins also differ from their corresponding wild-types in the exposure of their hydrophobic groups to the solvent, in the U state a protein of the size and composition of cytochrome *c* should have most of its hydrophobic side chains exposed to the solvent [11].

In the experiments reported here, both the wild-type and the mutant yeast cytochromes *c* appear as exceptions. First, for SC-iso1 *c* m is much lower than for other wild-type cytochromes *c* [7], which is consistent with the relatively low stability of the yeast protein [8,9], and could also be explained by a larger solvent exposure of its interior domain in the native state as compared with the other cytochromes *c*. Indeed, the crystal structure of yeast iso-1-cytochrome *c* [3] revealed significantly higher mobility of the areas surrounding W1 and adjacent domains in this protein than in the horse or tuna homologues. A second notable difference is that the Asn-52 $\rightarrow$ Ile mutation causes in SC-iso1 *c* an increase in m from 1.8 to 2.8 kJ (0.43 to 0.66 kcal) $\cdot$ mol $^{-2}$, and an increase in $\Delta G_{D,aq}$ from 6.1 to 12.1 kJ (1.46 to 2.90 kcal/mol, instead of the decreases observed for the single mutants of RNc. The stability enhancement brought about by the Asn-52 $\rightarrow$ Ile mutation in SC-iso-1 *c* was attributed mainly to increased hydrophobic interactions, together with stabilizing changes of the hydrogen-bonding pattern and side-chains packing in the surrounding area of the molecule [5]. Structurally, the most striking change due to the mutation is the elimination of W1 and the formation of hydrogen bonds between the side chains of Tyr-67 and Thr-78, and between the latter and a haem propionyl group [5]. Although the tertiary structure of RNc is not known, it is highly probable that it does not differ from the structures of the other vertebrate cytochromes *c* described in the literature, tuna [1] and horse [2]. If this assumption holds, then the major difference between RNc and SC-iso-1 *c* in this particular domain of the protein is that in the oxidized state of the yeast protein two hydrogen bonds are missing, one between Asn-52 and W1 and the other between Asn-52 and a haem propionyl group [3]. W1 is entirely between the side chain of Tyr-67 and

Thr-78; the hydrophobicity of this area increases, which probably explains the increase in conformational stability observed [5] and corroborated in the present study. It is not known whether a similar loss of W1 accompanies the Asn-52 $\rightarrow$ Ile mutation of RNc; if indeed it occurs, then the absence of W1 should eliminate the favourable contribution of the Asn-52–W1 hydrogen bond, which holds the hydrophilic Asn-52 side chain in the protein interior. This, in turn, may increase the solvent accessibility of the surrounding area, which would explain the lower values of both m and $\Delta G_{D,aq}$ that we observed.

Although the structural causes for these differences require further investigation, it is already apparent that the dissimilarities between the wild-type yeast iso-1 cytochrome *c* and those of vertebrates, represented here by the rat protein, are reflected in their different responses to the Asn-52 $\rightarrow$ Ile mutation.

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