Effects of mutating Asn-52 to isoleucine on the haem-linked properties of cytochrome *c*

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Asn-52 of rat cytochrome *c* and baker's yeast iso-1-cytochrome *c* was changed to isoleucine by site-directed mutagenesis and the mutated proteins expressed in and purified from cultures of transformed yeast. This mutation affected the affinity of the haem iron for the Met-80 sulphur in the ferric state and the reduction potential of the molecule. The yeast protein, in which the sulphur–iron bond is distinctly weaker than in vertebrate cytochromes *c*, became very similar to the latter: the pK_a of the alkaline ionization rose from 8.3 to 9.4 and that of the acidic ionization decreased from 3.4 to 2.8. The rates of binding and dissociation of cyanide became markedly lower, and the affinity was lowered by half an order of magnitude. In the ferrous state the dissociation of cyanide from the variant yeast cytochrome *c* was three times slower than in the wild-type. The same mutation

had analogous but less pronounced effects on rat cytochrome *c*: it did not alter the alkaline ionization pK_a nor its affinity for cyanide, but it lowered its acidic ionization pK_a from 2.8 to 2.2. These results indicate that the mutation of Asn-52 to isoleucine increases the stability of the cytochrome *c* closed-haem crevice as observed earlier for the mutation of Tyr-67 to phenylalanine [Luntz, Schejter, Garber and Margoliah (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 3524–3528], because of either its effects on the hydrogen-bonding of an interior water molecule or a general increase in the hydrophobicity of the protein in the domain occupied by the mutated residues. The reduction potentials were affected in different ways; the E'_0 of rat cytochrome *c* rose by 14 mV whereas that of the yeast iso-1 cytochrome *c* was 30 mV lower as a result of the change of Asn-52 to isoleucine.

INTRODUCTION

Das and co-workers (1989) reported that the Asn-52 → Ile mutant of yeast iso-1 cytochrome *c* had a higher global stability than the wild-type protein, reflected by the higher temperatures and guanidinium ion concentrations required to denature it. These results were later confirmed (Hickey et al., 1991; Koshy et al., 1994), but it was also shown that the same mutation in rat cytochrome *c* had the opposite effect, decreasing its global stability (Koshy et al., 1994).

In both tuna (Takano and Dickerson, 1981) and horse (Bushnell et al., 1990) cytochrome *c*, Asn-52 is hydrogen-bonded to an interior water molecule, termed W-1 by Takano and Dickerson (1981), as are two other side chains, those of Tyr-67 and Thr-78, whereas in the ferric form of SC-iso1c only the latter two side chains are hydrogen-bonded to W-1 (Berghuis and Brayer, 1992). It was previously shown (Luntz et al., 1989) that changing Tyr-67 to phenylalanine in RNc increased the stability of the Met-80 sulphur–haem Fe bond, a result that was attributed to the possible influence of the mutation on the hydrogen-bonded structure involving W-1. As a corollary to this conjecture, it was further hypothesized that mutations of the other two residues that hydrogen-bond to W-1 would also cause similar increases in the stability of the methionine–iron co-ordination (Luntz et al., 1989).

As reported previously (Koshy et al., 1994), in spite of their

extensive homology, RNc and SC-iso1c respond differently to the Asn-52 → Ile mutation, which weakens the global stability of rat cytochrome *c*, whereas it strengthens that of the yeast protein. The present study shows that the local effects of the mutation are also different in the two proteins. Whereas the mutation increased the stability of the haem crevice of SC-iso1c and RNc, these local effects were quantitatively different in the two cytochromes, being stronger for SC-iso1c than for RNc. It is well known that the closed haem crevice structure (George and Lyster, 1958) of the SC-iso1c is distinctly weaker than the corresponding structures of vertebrate homologues (Aviram and Schejter, 1969; see also Margoliah and Schejter, 1966), and the present results indicate that the Asn-52 → Ile mutation imparts a local stability to the haem crevice of SC-iso1c, similar in magnitude to that of the wild-type vertebrate cytochromes *c*. Furthermore, the reduction potentials of the two cytochromes *c* are affected differently by the mutation, that of RNc being increased by 14 mV and that of SC-iso1c being decreased by 30 mV.

EXPERIMENTAL

Preparative procedures

The expression systems and purification protocols, as well as the methods for introducing site-directed mutations into the cytochrome *c* coding sequences, have been described previously (Luntz et al., 1989; Koshy et al., 1990; Margoliah et al., 1990;

Abbreviations used: W-1, the interior water molecule hydrogen-bonded to Tyr-67, Asn-52 and Thr-78; SC-iso1c, wild-type *Saccharomyces cerevisiae* iso-1 cytochrome *c*; RNc, wild-type rat cytochrome *c*; CYC1, the gene for yeast iso-1 cytochrome *c*; RNc-N52I, RNc carrying the Asn-52 → Ile mutation; SC-iso1c-N52I, SC-iso1c carrying the Asn-52 → Ile mutation; Y67F, mutant carrying phenylalanine at position 67 in place of tyrosine; P30A, mutant with an alanine instead of a proline at position 30; Y67F/P30A, corresponding double mutant.

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Schejter et al., 1992). Briefly, the rat cytochrome *c* pseudogene, RC9 (Scarpulla, 1984) coding sequence, which yields the precise amino acid sequence of rat cytochrome *c* but lacks the intron of the functional rat gene, was cloned behind the promoter region of the yeast iso-1 cytochrome *c* gene (*CYC1*). This chimaeric gene was then cloned into the yeast episomal shuttle vector, YEp13 (Broach et al., 1979). Similarly, the entire 2.4 kb *CYC1* gene was cloned into YEp13, as also described by Faye et al. (1981). These expression plasmids were transformed into yeast GM-3C-2 (α , *leu2-3*, *leu2-112*, *trp1-1*, *his4-519*, *cyc1-1*, *cyc3-1*) (Zitomer et al., 1979) which had no endogenous cytochrome *c* genes of its own, but contains all the other yeast respiratory genes. These transformations confer respiratory sufficiency to the cells, indicating that the plasmid-encoded cytochrome *c* gene was able to complement the cytochrome *c*-deficient phenotype of the yeast. Site-directed mutagenesis was carried out as described by Geisselsoder et al. (1987).

Large quantities of cytochrome *c* (120–1400 mg) were prepared by growing yeasts harbouring a given cytochrome *c* gene in Braun Biostat fermentors of 30, 50 or 300 litres capacity. Large inocula of yeast were introduced into relatively small volumes of media which used either 4% sucrose and 1% ethanol or 6% glycerol as carbon source and, as the culture approached stationary phase, fresh medium was gradually added to increase the volume of the culture. Our protocol (Koshy et al., 1992), adopted from Stewart et al. (1971), gives considerably higher yields of yeast and much larger amounts of cytochrome *c* per gram of yeast than when small inocula are added to large volumes of medium. The optimal growth and cytochrome *c* production conditions varied to some extent for the different mutants but with some experience could be readily adapted to each.

Cytochromes *c* were extracted from yeast as described by Sherman et al. (1968), the lysate cleared by centrifugation, the cells washed on the centrifuge and the combined lysates diluted so that the protein could be collected on a bed of CM-cellulose. Cytochrome *c* was eluted from the resin and the solution brought to 60–70% saturation with $(\text{NH}_4)_2\text{SO}_4$ (depending on the cytochrome *c* being purified) to precipitate contaminating proteins without precipitating the cytochrome *c*. After dialysis and a concentration step employing a minimal CM-cellulose column, recombinant cytochromes *c* were purified by h.p.l.c. on a sulphopropyl cation-exchange column (Waters SP 5PW; 21.5 mm $\times$ 15 cm) (Koshy et al., 1992). Both the homologous yeast iso-1 cytochrome *c* gene and the heterologous rat RC9 gene in our expression system yielded two chromatographic fractions. Fraction I was acetylated at the N-terminus, as in RNc-I and SC-iso1c-I, and fraction II was unblocked, as in RNc-II and SC-iso1c-II. Fraction II constituted about 75% of the total and was employed exclusively in the present study. All these cytochromes *c* were trimethylated at Lys-72 (Koshy, 1991; Koshy et al., 1992).

Rat (RNc-N52I) and yeast (SC-iso1c-N52I) mutant cytochromes *c* were spectroscopically identical with their wild-type homologues, except for small differences similar to those observed for the rat P30A/Y67F double mutant cytochrome *c* (Schejter et al., 1992). For example, the ferric SC-iso1c-N52I cytochrome *c* visible and Soret maxima were at 528 and 409 nm, and the near-i.r. band was at 696 nm, as compared with 530 nm, 410 nm and 695 nm respectively for the wild-type protein. In the ferrous form, the ratio of the absorbances at the α and γ band maxima was 1.81, as in the wild-type protein. The isosbestic point of the Soret bands of the oxidized and reduced cytochromes was at 408 nm. As for the wild-type cytochromes *c*, no CO binding was observed in either of the two mutant proteins.

Analysis of spectroscopic titrations at 695 nm

The 695 nm band of cytochrome *c* is due to binding of thioether sulphur to the low-spin iron of the haem protein (Schechter and Saludjian, 1967; Schejter et al., 1991), and disappears when this bond is broken (Wooten et al., 1981). The titrations performed by measuring the absorbance changes at 695 nm were treated as two-state processes, because the spectroscopic change represents the equilibrium between two differently liganded states:

where Im is the imidazole of His-18, (Met)S is the sulphur of Met-80 and K_{Cr} is the corresponding equilibrium constant. The nature of X is controversial [reviewed in Moore and Pettigrew (1990)], although recent evidence tends to support its identification as a lysine when the equilibrium is driven to the right by alkaline pH (Ferrer et al., 1993).

pH titrations

Optical spectroscopy was performed with Hitachi 557 and Cary 14 spectrophotometers. For the alkaline titrations, the ferric cytochromes *c* were dissolved in 0.1 M glycine/NaOH buffers. In the acid titrations, very small amounts of dilute HCl were added to solutions of the protein in 5 mM sodium phosphate buffer, pH 6.5. The pH values were read directly in the cuvettes with an Ingold combined glass microelectrode. When required, the data were analysed by using non-linear regression procedures (Fig. P, Version 5, from Biosoft, Cambridge, U.K.).

Kinetics and equilibria with cyanide

For the ferric state of rat, yeast and mutant cytochromes *c* the equilibria with cyanide were studied as described by George and co-workers (1967). To avoid complications arising from the effects of temperature on the sulphur-iron bond, the experiments were conducted at 20 °C. The kinetics of formation of the cyanide complexes of these ferric cytochromes *c*, at increasing concentrations of the ligand, were determined by measuring the increase in A_{418} or the decrease in A_{695} . The second-order rate constants were obtained by plotting the observed pseudo-first-order constants against total cyanide concentration. First-order dissociation kinetics were measured by observing the opposite optical changes after removal of the cyanide by passage of the cyanide-saturated cytochrome *c* solutions through a Sephadex G-25 column.

In the reduced state, cytochrome *c* does not bind cyanide (George and Tsou, 1952), but the ferrous complex can be formed by reducing the ferricytochrome *c*-cyanide complex with dithionite, and its dissociation can be subsequently followed (George and Schejter, 1964). This dissociation reaction was studied spectrophotometrically at various temperatures and the activation parameters were evaluated.

Reduction potentials

The reduction potentials of the ferric/ferrous cytochrome *c* couples were determined using the spectroscopic method of mixtures as described elsewhere (Margalit and Schejter, 1973).

Computer modelling

Modelling of the cyanide complex of ferric cytochrome *c* was

carried out on the personal IRIS computer graphics system (Silicon Graphics), using the program package INSIGHT II, version 2.1.0 (BIOSYM). To simulate cyanide binding, we used nitrile ($\text{N}\equiv\text{C}-\text{H}$), one of the standard groups provided by the program, and the atomic co-ordinates of the 'inner molecule' of ferric tuna cytochrome *c* (Takano and Dickerson, 1981). It was assumed that the haem conformation remains the same in the cyanide–cytochrome *c* complex as in the native protein, and that the N and C atoms in cyanide and the Fe atom of haem are on a straight line, normal to the haem plane. The Fe–cyanide distance was taken to be 0.2 nm, an arbitrary value in the region of 0.195–0.315 nm measured for a variety of metals complexed to cyanide (Britton, 1967). Zn, the most similar to Fe among the metals studied, gives a distance of 0.198 nm. The images were printed out on a laser printer in colour and then digitized to produce the black and white versions given in Figure 2.

The atomic co-ordinates of the structures of the various cytochromes *c* employed in modelling studies and for examining various atomic distances or the conformation of various side chains were taken from the Protein Data Bank (Bernstein et al. 1977) for tuna (Takano and Dickerson, 1981) and yeast iso-1 (Berghuis and Brayer, 1992) ferricytochromes *c* and from R. G. Sanishvili, K. W. Volz, F. M. Westbrook and E. Margoliash (unpublished work) for the horse protein.

RESULTS

Titration of the alkaline ionizations

The disappearance of the 695 nm band in moderately alkaline pH, in the type-III to type-IV transition of the ferric protein (Theorell and Åkesson, 1941), is observed at a distinctly lower pH in yeast iso-1 cytochrome *c* than in the mammalian cytochromes *c* (Aviram and Schejter, 1969). The recombinant SC-iso1c, like the non-recombinant protein, had a pK_a of 8.3, whereas the pK_a of SC-iso1c-N52I was 9.4 (Table 1). This indicates a considerable increase in the strength of the Met-80 sulphur–haem Fe bond. This change was not observed in the RNC-N52I mutant, the pK_a being 9.5, whereas the RNC had a pK_a of 9.6 (Table 1).

Titration of the acidic ionizations

This pH-linked transition of ferric cytochrome *c* (Theorell and Åkesson, 1941) is an equilibrium between several species of the protein (Dyson and Beattie, 1982; Moore and Pettigrew, 1990). In SC-iso1c the midpoint of this titration is observed at pH 3.4 (Aviram and Schejter, 1969), whereas in higher eukaryotes it is at pH 2.5 (Margoliash and Schejter, 1966).

In our titrations, the observed midpoints were at pH values of

Table 1 Effects of mutations on the pH-dependent equilibria and the reduction potentials of cytochrome *c*

Protein	Acid pK_a	Alkaline pK_a	E'_0 (mV)
RNC	2.8	9.6	259
RNC-N52I	2.2	9.5	273
SC-iso1c	3.4	8.3	268
SC-iso1c-N52I	2.7	9.4	238

Figure 1 Plot of the observed rate constants for the formation of cytochrome *c*–cyanide complexes as a function of the total ligand concentration

Reactions were measured in potassium phosphate buffer, pH 7.0, at 25 °C. ●, RNC; ○, RNC-N52I; ■, SC-iso1c; □, SC-iso1c-N52I.

2.8 and 3.4 for RNC and SC-iso1c respectively. In both proteins, the Asp-52 → Ile mutation caused a decrease by 0.6–0.7 pH unit, indicating that the mutation had increased the strength of the sulphur–iron bond by about 3.9 kJ/mol (Table 1).

Reactions with cyanide

In the ferric state, the binding of cyanide by RNC was barely altered by the mutation, but that of SC-iso1c-N52I was notably different from the parent wild-type protein. This is graphically shown in Figure 1, where the ligand-concentration-dependences of the observed binding rates are compared.

The kinetic and equilibrium constants for the reaction of ferricytochrome *c* with HCN at pH 7.0 and 25 °C are listed in Table 2. The observed rate of complex formation for RNC was slightly lower than that for SC-iso1c, but a markedly lower dissociation rate of the RNC–cyanide complex resulted in a higher affinity of the rat protein for the ligand (Table 2). The effects of the Asn-52 → Ile mutation on the kinetic and equilibrium constants of the reaction of RNC with cyanide were negligible, but for SC-iso1c the rates of formation and dissociation became distinctly slower, resulting in a less favourable binding constant by about 2.9 kJ/mol.

The dissociation rates of the ferrocyanide complexes are reported in Table 3, together with the corresponding activation parameters. For the two proteins, the Asn-52 → Ile

Table 2 Effects of the Asn-52 → Ile mutation on the kinetic and equilibrium constants for the reaction of ferricytochrome *c* with cyanide in 0.1 M potassium phosphate buffer, pH 7.0, at 25 °C

k_f is the second-order rate constant for the forward reaction leading to the formation of the ferricytochrome *c*–cyanide complex, k_b the first-order rate constant for the back reaction leading to its dissociation and K the equilibrium constant for the reaction.

Cytochrome	k_f ($\text{mM}^{-1} \cdot \text{s}^{-1}$)	k_b (s^{-1})	K (M^{-1})
RNC	2.0×10^{-4}	3.8×10^{-4}	5.3×10^{-1}
RNC-N52I	2.2×10^{-4}	4.3×10^{-4}	5.1×10^{-1}
SC-iso1c	3.3×10^{-4}	1.7×10^{-3}	1.9×10^{-1}
SC-iso1c-N52I	8.4×10^{-6}	1.4×10^{-4}	6.1×10^{-2}

Table 3 Observed rate constants, energies and entropies of activation for the dissociation of ferrocyclochrome *c*-cyanide complexes in 0.1 M potassium phosphate buffer, pH 7.0

k_b is the first-order rate constant for the back reaction resulting in the dissociation of the ferrocyclochrome *c*-cyanide complex, E_a is the energy of activation of this reaction and ΔS its entropy of activation.

Cytochrome	k_b (25 °C) (s ⁻¹)	E_a (kJ/mol)	ΔS (kJ · mol ⁻¹ · K ⁻¹)
RNc	4.72×10^{-3}	92.0	2.8
RNc-N52I	4.90×10^{-3}	99.5	8.9
SC-iso1c	3.7×10^{-3}	97.8	7.0
SC-iso1c-N52I	1.2×10^{-3}	114.5	18.2

mutation increased both the energy and the entropy of activation, but in different proportions. As a result of these changes the dissociation rate of RNc-N52I-cyanide remained identical with that of RNc-cyanide, but for the yeast cytochrome *c* the dissociation of the cyanide complex of the mutant protein was three times slower than that of the wild-type cytochrome.

Reduction potentials

The mutation had different effects on the two cytochromes *c*: E'_0 decreased for SC-iso1c from 268 to 238 mV, whereas it increased for RNc from 259 to 273 mV (Table 1). The values for the yeast protein obtained by other authors (Langen et al., 1992) are not identical with ours, but they follow the same diminishing trend from wild-type to mutant, from 275 to 220 mV.

DISCUSSION

A central problem in the study of protein stability is the impossibility of obtaining direct measures for the stabilities of individual interactions (Creighton, 1984). Nevertheless, certain intramolecular bonds that can be identified and reversibly broken are amenable to direct investigation of their stabilities, as exemplified by disulphide bonds (Goldenberg and Creighton, 1984). In ferrocyclochrome *c* there is a well-understood correlation between the Met-80 sulphur-iron bond, which can be reversibly broken in a number of different ways, and the 695 nm band of the optical spectrum, whose position and intensity depend on the nature of the axial iron ligands (Shechter and Saludjian, 1967; Schejter et al., 1991).

The main objective of this investigation was to study the effect of the Asn-52 → Ile mutation on the stability of the Met-80 sulphur-iron bond that is an essential element of the structure and function of cytochrome *c*. Several reactions of ferrocyclochrome *c* have in common an equilibrium between two states, in which the S-Fe bond is either intact or broken [eqn. (1)]. These can be studied by optical spectroscopy because the processes in which the S-Fe bond is broken and re-formed are directly observable through the 695 nm band.

In this regard, it is of interest to speculate about the possible effect on W-1 of breaking the S-Fe bond; this can be exemplified by the cyanide-cytochrome *c* complex. Employing computer graphics and the atomic co-ordinates of tuna ferric cytochrome *c*, it was found that when cyanide is bound to the haem iron, it occupies space very close to the side chain of Met-80, causing displacement of the latter from its original position, without

seriously disturbing the back-bone conformations of nearby residues (Figures 2a and 2b). The bold circle in Figure 2(a) represents the trajectory of the Met-80 C_γ atom in free rotation around the C_α-C_β bond. Most of this circle (the bigger arc between A and B) is inaccessible to C_γ because of close contacts with the cyanide, pyrrole ring III and the carbonyl of Met-80. The only accessible area is within the smaller arc AB. Even in this location the C_γ atom is restrained because of proximity of the back bone, and thus may cause some minor conformation adjustments in its immediate vicinity. Depending on the position of the C_γ atom in the AB arc, the rest of the side chain may or may not require rotation of the side chain of Thr-78 by about 180°. In either case the methyl group of methionine displaces the internal water molecule by occupying the same space (Figure 2b).

In this new position of the Met-80 side chain, its sulphur atom can hydrogen bond to the hydroxyl group of Tyr-67. Although the distance between the side chains of Met-80 and Thr-78 is compatible with a hydrogen bond, their orientations make it less likely. This might indicate a possible preference for the Thr-78 side chain to rotate by about 180°, where it could switch hydrogen bonds from the anterior haem propionic acid group to its own amide group. As a result, hydrophobic interaction between the methyl groups of Thr-78 and Met-80 is favoured, stabilizing the local conformation. Even though the side chain of Thr-78 is no longer hydrogen-bonded to the anterior haem propionate, the latter, after very small adjustments, could form a hydrogen bond with the amide group of Lys-79, the distance between the corresponding oxygen and nitrogen atoms being 0.3 nm. Given that the Asn-52 → Ile mutation in SC-iso1c also appears to displace W-1 [A. M. Berghuis and G. D. Brayer, unpublished work; cited by Hickey et al. (1991)], it is possible that the Thr-78 side chain is rotated in these mutants, as it is likely to be in the cyanide complex.

An effective experimental way of gauging the stability of the S-Fe state is by breaking the sulphur-iron bond with an exogenous ligand (George and Tsou, 1952; George et al., 1967; Schejter and Aviram, 1969). At low ligand concentrations the rates of the reactions of ferrocyclochrome *c* with cyanide (George and Tsou, 1952) and imidazole (Schejter and Aviram, 1969) depend on the concentrations of the ligands. However, at high ligand concentrations these reactions reach a limiting rate constant of 30–60 s⁻¹ which has been interpreted as representing the S_N1 opening of the crevice, namely the cleavage of the sulphur-iron bond (Sutin and Yandell, 1972).

Our rate studies at low cyanide concentrations (Table 2) show that the Asn-52 → Ile mutation had no effect on RNc, but noticeably changed the reactivity of SC-iso1c. Formation of the complex was 40 times slower for SC-iso1c-N52I than for the wild-type cytochrome, and its dissociation was about ten times slower, resulting in a decrease in the affinity for cyanide of 2.9 kJ/mol, which can be attributed to a corresponding increase in the stability of the native crevice.

Ligands do not bind ferrocyclochrome *c* because of the unusually large stability of the ferrous haem crevice (Margolias and Schejter, 1966), but the dissociation of ferrocyclochrome *c*-cyanide can be observed by reducing the ferric complex with dithionite (George and Schejter, 1964). As in the reactions that were studied in the ferric state, in the ferrous state, the N52I mutation had a significantly stronger effect on SC-iso1c-II than on RNc. For both proteins the energies and entropies of activation were larger for the mutant than for the wild-type (Table 3).

The rate-limiting process observed in the dissociation is best interpreted as the re-formation of the closed-crevice structure (George and Schejter, 1964). It appears reasonable to attribute

Figure 2 (a) Stereodiagram of the 'lower left' area of the cytochrome *c* showing the haem and the internal water molecule viewed from the 'left rear side' of the molecule [according to Takano and Dickerson (1981)] and (b) proposed effects of the formation of the cyanide-cytochrome *c* complex

(a) The polypeptide back bone is represented by bold lines, the thin lines correspond to the side chains and the haem prosthetic group, and the broken lines represent the hydrogen bonds. Cyanide is shown by its van der Waals surface. For simplicity, the side chain of Met-80 is truncated at the C_γ atom and is moved from its original position. The hydrogen bonds indicated are those given by Takano and Dickerson (1981). (b) The open lines correspond to the side-chain conformations in the original structure that have been changed as a result of complex-formation, as shown by the modelling study. The proposed new hydrogen bonds formed are indicated by the broken lines carrying numbers corresponding to the distance (in nm) between donor and acceptor atoms.

the increase in activation energy to effects caused by the mutation on the environment of the altered side-chain, which is precisely the portion of the protein in which the Met-80 side chain appears to be nestled, after being removed from iron co-ordination. The Asn-52 → Ile mutation should increase the hydrophobicity of this protein region, similarly to the previously described effect of the Tyr-67 → Phe mutation on rat cytochrome *c* (Luntz et al., 1989). Thus, in the RNC-N52I-II mutant protein, the hydrophobic Met-80 side chain should be more strongly anchored in its position in the X-Fe state, and consequently it should require

a larger energy of activation for its removal from that position, and subsequent return to ligation of the iron.

A possible explanation for the differences in the reaction of cyanide with SC-iso1c and RNC could be related to different conformations of Asn-52 in these proteins. In ferric SC-iso1c (Berghuis and Brayer, 1992), the rotation angle around the C_α and C_β bond, termed χ_1 , is 189° according to the convention used by McGregor et al. (1987). In tuna and horse ferric cytochromes *c* the same angle is 202° . As a result the Asn-52 side chain in ferric SC-iso1c is not bound to the internal water molecule but forms

hydrogen bonds with the hydroxyl group of Ser-40 and the backbone amide of Gly-41. On the other hand, in the ferric vertebrate proteins the Asn-52 side chain is hydrogen-bonded to W-1 and to the propionyl side chain of pyrrole ring IV. Thus the size of the cavity occupied by W-1 is larger in yeast than in the vertebrate cytochromes *c*. It could therefore be expected that the postulated movement of the Met-80 side chain and the consequent displacement of W-1 forced by cyanide binding to the haem iron would be easier in yeast than in vertebrate ferricytochromes *c*. Indeed, in the latter, this displacement of the internal water molecule leads to its removal from the interior of the protein, as shown by modelling, whereas in SC-iso1c, adoption of a new position by W-1 in the same cavity cannot be excluded. Even if this is not the case, the expulsion of W-1 is easier in the yeast protein where it is held by two hydrogen bonds, as compared with three in the vertebrate cytochromes *c*.

In the N52I mutants, the $C_{\gamma 2}$ methyl group of isoleucine would force the rest of the side chain to the position occupied by that of asparagine in the wild-type vertebrate proteins. In RNc-N52I, assuming that W-1 has been expelled as it is from the SC-iso1c-N52I mutant (Hickey et al., 1991), the only effect would be an increase in hydrophobicity in this region. In the yeast cytochrome *c* mutant, in addition to this effect, there will be a decrease in the size of the cavity originally occupied by W-1. Remarkably, the residues forming this cavity, other than Asn-52, in the vertebrate wild-type proteins are closer together in the yeast protein. For example, the distance between the $O_{\gamma 1}$ of Thr-78 to the OH of Tyr-67 is 0.443 nm in yeast, 0.466 nm in horse and 0.477 nm in tuna ferricytochrome *c*. Similarly, the distance between the OH of Tyr-67 and the N of pyrrole ring IV is 0.367, 0.389 and 0.388 nm in these three proteins respectively. Therefore, if Ile-52 has indeed a similar conformation in the yeast and vertebrate mutant proteins, the cavity remaining in the former would be even smaller than in the latter. It is likely that these differential conformational effects of the mutation underlie the differences in reactivity with cyanide given in Figure 1 and Table 2. The particularly small cavity of the yeast mutant protein is compatible with the dramatic decrease in the rate of cyanide binding as compared with, not only the SC-iso1c but also both RNc and RNc-N52I, since a small cavity would make it more difficult for the Met-80 side chain to be displaced from Fe binding. Similarly, the larger size of the cavity in SC-iso1c than that in the RNc could be responsible for the greater cyanide reactivity of the former, while the small difference in reactivity between RNc and RNc-N52I represents the apparently small change in cavity size resulting from the similar conformations of the Asn-52 and Ile-52 side chains suggested above.

The structural differences between the yeast and vertebrate proteins discussed above probably also underlie the variability of the pH equilibria discussed in this paper, and the quantitatively different effects of mutations in this area in cytochromes *c* of different species. Thus the mid-point of the alkaline ionization depends on the species of origin; for most cytochromes *c* it is observed at pH 9.4, but it may vary from 8.4 to 10.4 (Brautigan et al., 1977).

While mutation of RNc Tyr-67 to phenylalanine raised the alkaline pK_a by 1.2 pH units (Luntz et al. 1989), the similar expected change for RNc-N52I failed to take place (Table 1). For SC-iso1c, however, for which the low value of 8.3 was attributed to weakness of the S-Fe bond (Aviram and Schejter, 1969), the N52I mutation raised the alkaline pK_a to 9.4, a typical value for vertebrate cytochromes *c*. These different effects of the mutation on the two proteins were not observed for the acidic ionization, a more complicated process than the alkaline ionization but still involving the breakage of the S-Fe bond (Dyson and Beattie,

1982; Moore and Pettigrew, 1990). The observed acidic pK_a is 2.8 for RNc and 3.4 for SC-iso1c, indicating again a weaker S-Fe bond in the latter. In both Asn-52 $\rightarrow$ Ile mutants this pK was lowered, by 0.6 and 0.7 pH unit respectively (Table 1), confirming the expected stabilization of the S-Fe bond.

Another property in which rat and yeast cytochromes *c* differed in their responses to the Asn-52 $\rightarrow$ Ile mutation is the reduction potential: E'_0 of RNc rose from 259 to 273 mV, indicating an increase in the stability of the reduced state relative to the oxidized state, it fell for SC-iso1c from 268 to 238 mV. In view of the multiplicity of parameters that regulate the reduction potential of haem proteins (Moore and Pettigrew, 1990), a detailed discussion of these results is necessarily an oversimplification. However, it is interesting to point out that RNc-N52I is outstanding in that it is the only cytochrome *c* mutant so far reported for which an increase in the reduction potential has been measured.

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