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Rates and Energetics of Tyrosine Ring Flips in Yeast Iso-2-cytochrome *c*†

Barry T. Nall* and Efrain H. Zuniga‡
Department of Biochemistry, University of Texas Health Science Center, San Antonio, Texas 78284
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ABSTRACT: Isotope-edited nuclear magnetic resonance spectroscopy is used to monitor ring flip motion of the five tyrosine side chains in the oxidized and reduced forms of yeast iso-2-cytochrome *c*. With specifically labeled protein purified from yeast grown on media containing [3,5-^{13}C]tyrosine, isotope-edited one-dimensional proton spectra have been collected over a 5–55 °C temperature range. The spectra allow selective observation of the 10 3,5 tyrosine ring proton resonances and, using a two-site exchange model, allow estimation of the temperature dependence of ring flip rates from motion-induced changes in proton line shapes. For the reduced protein, tyrosines II and IV are in fast exchange throughout the temperature range investigated, or lack resolvable differences in static chemical shifts for the 3,5 ring protons. Tyrosines I, III, and V are in slow exchange at low temperatures and in fast exchange at high temperatures. Spectral simulations give flip rates for individual tyrosines in a range of one flip per second at low temperatures to thousands of flips per second at high temperatures. Eyring plots show that two of the tyrosines (I and III) have essentially the same activation parameters: $\Delta H^{\ddagger}$ = 28 kcal/mol for both I and III; $\Delta S^{\ddagger}$ = 42 cal/(mol·K) for I, and $\Delta S^{\ddagger}$ = 41 cal/(mol·K) for III. The remaining tyrosine (V) has a larger enthalpy and entropy of activation: $\Delta H^{\ddagger}$ = 36 kcal/mol, $\Delta S^{\ddagger}$ = 72 cal/(mol·K). Tentative sequence-specific assignments for the tyrosines in reduced iso-2 are suggested by comparison to horse cytochrome *c*. For oxidized iso-2, five resonances are observed at high temperatures, suggesting flip rates for all five tyrosines sufficient to average static chemical shift differences. At lower temperatures, there is evidence of intermediate and slow flipping for some of the rings, but spectral simulations have not been possible because of the complexity of the isotope-edited spectra.

The investigation of internal motions in proteins provides an important link between experiment and theory for protein-mediated phenomena. Measurement and modeling of fundamental intramolecular rate processes is of importance in understanding the internal movement and rearrangement of protein segments that occurs together with enzyme catalysis, allostery, and molecular recognition. Two experimental approaches are particularly valuable: exchange of labile protons with solvent protons (HX)[1] and the rotational motion of aromatic side chains (ring flips) (Wagner, 1983). HX allows measurement of the location and equilibrium constants for protein breathing modes but only infrequently gives a direct measure of rates (Englander & Kallenbach, 1984). On the other hand, accurate rate information is obtained for protein fluctuations involving aromatic ring flips (Campbell et al., 1976; Wuthrich & Wagner, 1975; Wuthrich, 1979). The fluctuations in local environment probed by aromatic ring flips are in the time range of important biochemical processes (1 ms to 1 s) such as enzyme catalysis, protein conformational changes, and protein folding.

Despite its fundamental importance, protein internal dynamics as probed by aromatic ring motion have been studied in detail for a limited number of systems: principally BPTI (Snyder et al., 1975; Wagner, 1983, 1987; Wagner et al., 1976), alkaline phosphatase (Hull & Sykes, 1975), and horse cytochrome *c* (Campbell et al., 1976). This is largely because of the difficulty of obtaining suitable NMR spectra. The detailed line-shape analysis needed to extract rate constants

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* Address correspondence to this author at the Department of Biochemistry, University of Texas Health Science Center, 7703 Floyd Curl Dr., San Antonio, TX 78284-7760.
‡ Present address: Department of Biochemistry and Molecular Biology, University of Texas Medical School, Houston, TX 77225.

[1] Abbreviations: iso-2, iso-2-cytochrome *c* from the yeast *Saccharomyces cerevisiae*; BPTI, basic pancreatic trypsin inhibitor; NMR, nuclear magnetic resonance; D_2O, deuterium oxide; pD, uncorrected pH meter reading for a deuterium oxide solution; HX, labile proton exchange with either deuterium or tritium; s, seconds; ms, milliseconds; *k*, rate constant for ring flips (s^{-1}); Hz, hertz (s^{-1}).

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from chemically exchanging systems places stringent requirements on resolution and signal to noise ratio. Of current interest is how mutations might alter ring flip motion. With the exception of chemically modified forms of BPTI (Wagner et al., 1979), there is little experimental or theoretical information available on mutational effects on protein internal dynamics. Yeast iso-2-cytochrome *c* is a useful model system for investigating mutation-induced changes in ring flip motion. The protein can be labeled in vivo and, together with yeast iso-1-cytochrome *c*, is among the most highly developed systems for manipulation of protein structure by molecular genetic methods (Hampsey et al., 1986; Pielak et al., 1985; Sherman & Stewart, 1978; White et al., 1987). Here we show that isotope-edited NMR spectroscopy can be used to obtain detailed information about the internal motions of aromatic residues in normal iso-2.

Materials and Methods

Growth of Yeast and Tyrosine Labeling. Growth of yeast and protein purification and characterization have been described previously (Nall & Landers, 1981; Wood et al., 1988). Yeast strain D870-3D (*a* cyc1 CYC7-H1 aro7-1 his 5-2 rad1 trp1-1 ilv1-1 trp2), a gift from Fred Sherman (University of Rochester School of Medicine and Dentistry), was grown on SD medium (0.67% Bacto-yeast nitrogen base without amino acids, 2% dextrose). The medium was supplemented with all amino acids except tyrosine in the recommended amounts (Sherman et al., 1974). The tyrosine supplement was 20 mg/mL of 99% [3,5-^{13}C]tyrosine obtained from the NIH Stable Isotope Resource at Los Alamos, New Mexico. This is two-thirds of the usual amount of tyrosine added to minimal media (30 mg/L) but gives full cell yields, indicating that tyrosine is not limiting for growth. From a 10-L fermentation, about 70 g of cell paste is obtained (wet weight) which gives a 3.5–5.2 mg of specifically labeled iso-2 on purification (White et al., 1987).

NMR Measurements. Samples were prepared from lyophilized protein at a concentration of 0.5–0.8 mM in 99.7% D_2O (Cambridge Isotopes)/0.1 M sodium phosphate, pD 6.0. Sample pD (meter readings, not corrected for isotope effects) was adjusted with deuterium chloride. The oxidation state of samples was verified by ^{1}H NMR spectroscopy. In most cases, samples were largely in the oxidized state after preparation. Oxidized cytochrome *c* samples containing small amounts of contaminating reduced protein were fully oxidized by heating the sample to 50–55 °C for 5–10 min in the spectrometer. Reduced samples were prepared by bubbling N_2 through the sample for several minutes and adding a small amount of solid sodium dithionite.

Spectra were obtained on a JEOL GX-270WB spectrometer with a variable-temperature 5-mm ^{1}H probe fitted with a tunable X-nucleus decoupling coil. Proton ($\pi/2$) pulse widths were 7.5–10.0 μs. Band-pass filters (Wavetek Indiana, Inc.) were inserted at the probe connections for the ^{1}H (observe) and ^{2}H (lock) radiofrequency channels to block spurious signals generated by ^{13}C (X-nucleus) decoupling. The pulse sequence for isotope-edited difference spectra is a one-dimensional analogue of a scheme used for 2D heteronuclear correlation spectroscopy (Bax et al., 1983) and is similar to *J*-modulated internuclear difference spectroscopy (Griffey et al., 1985a). The method, which we call *J*-echo difference spectroscopy, is a classic proton spin-echo experiment, ^{1}H-($\pi/2$)-Δ-^{1}H(π)-Δ-acquire, with on/off-resonance ^{13}C(π)-pulses coinciding with the ^{1}H(π) pulse. The delay, Δ, is set equal to $1/2J_{CH}$ = 3.2 ms for tyrosine ring protons. Signals acquired with ^{13}C(π) on- and off-resonance pulses are averaged separately and, after completion of the experiment, Fourier transformed, phased, and subtracted to give the isotope-edited spectrum. Decoupled spectra are obtained by using the same pulse sequence with broad-band ^{13}C irradiation during the acquisition time. Typically, 6K transients each are acquired on- and off-resonance with a 30-kHz bandwidth, 0.544-s acquisition time, and total pulse recycle times of 0.61–0.80 s.

Isotope-detected NOE double difference spectra (Figures 4A,B and 5) are obtained essentially as described (Weiss et al., 1986) except that ^{13}C preirradiation is used to collapse the J_{CH} doublet and direct the selective ^{1}H preirradiation to protons attached to ^{13}C atoms (Griffey et al., 1985b). For each of four pulse sequences (Figure 4A), 2K transients were averaged by using a 30-kHz bandwidth and 100 ms of proton (on- or off-resonance) preirradiation. The spectra were taken at 20 °C with an acquisition time of 0.544 s and pulse recycle times of 1.7 s.

Data Analysis. Spectral simulations have been carried out by using a model of two-site chemical exchange (Gutowsky et al., 1953; Hahn & Maxwell, 1952) describe by Slichter (1978). The solution to the Bloch equations for an exchanging system (see Slichter, appendix F) gives a total complex magnetization of

$$M_x + iM_y = \{i\gamma H_1 M_0 (1/k)[2 + (1/2k)(\alpha_a + \alpha_b)]\}/ \{[1 + \alpha_a/k][1 + \alpha_b/k] - 1\}$$

where

$$\alpha_a = \Delta\omega^a{}_{1/2}/2 + i(\omega_0 - \omega - \delta\omega/2)$$

$$\alpha_b = \Delta\omega^b{}_{1/2}/2 + i(\omega_0 - \omega + \delta\omega/2)$$

k is the exchange rate, ω_0 the angular frequency at the midpoint between the (rigid lattice) resonance frequencies for the exchanging sites, $\delta\omega$ the (rigid lattice) angular frequency separation of the exchanging sites, $\Delta\omega^{a,b}{}_{1/2}$ the line width at half-height (in the absence of exchange) for resonance "*a*" or "*b*", respectively, and ω the angular frequency. The factor $\gamma H_1 M_0$ can be taken as a constant that determines the magnitude but not the shape of the signal. The absorption mode signal is proportional to the imaginary part of the complex magnetization. Assuming equal line widths for resonances "*a*" and "*b*", $\Delta\omega^a{}_{1/2} = \Delta\omega^b{}_{1/2} = \Delta\omega_{1/2}$, and the line shape, $S(\omega)$, is given by

$$S(\omega) = (AC + BD)/(A^2 + B^2)$$

where

$$A = \Delta\omega_{1/2}{}^2/4k^2 + \Delta\omega_{1/2}/k - (1/k)(\omega_0 - \omega - \delta\omega/2)(\omega_0 - \omega + \delta\omega/2)$$

$$B = (2/k + \Delta\omega_{1/2}/k^2)(\omega_0 - \omega)$$

$$C = 2/k + \Delta\omega_{1/2}/2k^2$$

$$D = (\omega_0 - \omega)/k^2$$

Subspectra for each of the five spin systems were computed and added together to simulate the total spectrum. Calculations were carried out on a MacIntosh SE computer with a program written in Microsoft QuickBasic. Flip rates (k) were estimated by comparing observed (^{13}C decoupled) and simulated spectra over the temperature range where the line shape was a sensitive function of temperature. A 25–45 °C temperature range was used for spin systems I and III and a 20–45 °C range for spin system V. Least-squares fits to the Eyring equation (Figure 7) were used to extrapolate k for simulations outside the 20–45 °C temperature range. Error limits for k values, obtained by spectral simulations, were taken as those

which gave simulated spectra clearly distinguishable from observed spectra. Actual errors may be somewhat smaller. To test the methodology and the consistency of the data, flip rates obtained for decoupled spectra were used to simulate coupled spectra. For coupled spectra, the line-shape function for each spin system was replaced by two line-shape functions separated by J_{CH} = 157 Hz and centered at ω_0. Total (coupled) spectra were obtained by summing subspectra from all five spin systems.

The thermodynamic reaction rate parameters have been obtained by least-squares fits to plots of $\Delta G^{\ddagger}/T$ versus $1/T$ according to the equation:

$$\Delta G^{\ddagger}/T = R \ln \{[k_b T/h][1/k]\} = \Delta H^{\ddagger}/T - \Delta S^{\ddagger}$$

where $\Delta G^{\ddagger}$, $\Delta H^{\ddagger}$, and $\Delta S^{\ddagger}$ are the activation free energy, activation enthalpy, and activation entropy, respectively; R, the gas constant; k_b, Boltzmann's constant; h, Planck's constant; and T, temperature in degrees kelvin. The flip rates, k, are from spectral simulations over the temperature range where the line shape is a strong function of temperature: 25–45 °C for spin pairs I and III, 20–45 °C for spin pair V.

Results

Reduced Iso-2: Isotope-Edited Spectra. Figure 1 gives isotope-edited spectra of reduced iso-2 over a 50 °C temperature range. Comparison of the ^{13}C-decoupled (Figure 1A) and coupled (Figure 1B) spectra shows the expected 157-Hz J_{CH} splitting of spectral lines in the coupled spectra. At the ^{1}H frequency of 270 MHz used for these experiments, the J_{CH} splitting is comparable to the chemical shift range of the tyrosine 3,5 ring protons. Thus, the coupled spectra are more complex with greater resonance overlap. Nevertheless, for both coupled and decoupled spectra, there are indications of slow exchange at lower temperatures which passes to intermediate exchange (intermediate temperatures) and finally fast exchange (high temperatures). The clearest example is in the 7–8 ppm region where a classic example of two-site exchange is observed on the left-hand edge of the resonance packet. In the 5–6.5 ppm region, there are indications of motional broadening of two resonances (5–35 °C) and motional narrowing of a resonance just above 5 ppm (35–55 °C). This suggests that at least three of the five tyrosines pass from slow to fast exchange over the temperature range monitored in these experiments.

Oxidized Iso-2: Isotope-Edited Spectra. Figure 2 shows a similar series of spectra for the oxidized protein. Decoupled (Figure 2A) and coupled (Figure 2B) spectra have been collected over a 40 °C temperature range (5–45 °C). With the possible exception of the high-temperature region (35–45 °C), the resonance lines tend to be broader and less well resolved than for the reduced protein. This is probably because of combined paramagnetic and motional broadening of the spectra. In the 35–45 °C temperature range, all five J_{CH} doublets are observed (Figure 2B). This is as expected. There should be one doublet for each tyrosine assuming all are in fast exchange. Fast flip rates are expected for all rings at high temperature since 45 °C is only slightly below the temperature-induced unfolding transition zone for oxidized iso-2 (T_m = 53–54 °C, Dumont et al., 1990; Liggins, Nall, and Sturtevant, unpublished results). The decoupled spectra (Figure 2A) show greater than expected distortion of the relative intensities of resonances compared to the coupled spectra (Figure 2B). In addition, the 5 and 10 °C spectra cannot be phased satisfactorily, suggesting instrumental problems at lower temperatures. Wide spectral bandwidths were used, so it is unlikely that paramagnetically shifted resonances have folded into the spectra. These effects are reproducible but remain unexplained.

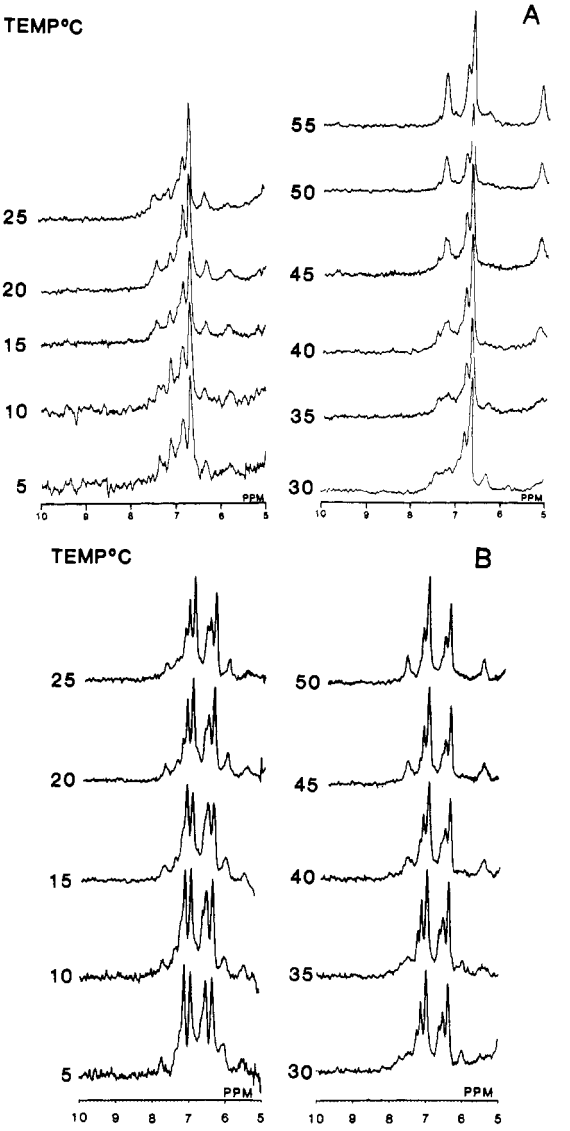

FIGURE 1: Temperature dependence of ^{13}C-decoupled (A) and coupled (B) isotope-edited proton NMR spectra of reduced [3,5-^{13}C]tyrosine iso-2-cytochrome *c*. Spectra, obtained by *J*-echo difference spectroscopy, show resonances from protons with J_{CH} coupling to ^{13}C atoms (3,5 tyrosine ring protons) while resonances from other protons are edited out. Samples contain 0.5–0.8 mM cytochrome *c* dissolved in 99.7% D_2O/0.1 M sodium phosphate, pD 6.0, in a 5-mm NMR tube.

At least two resonances, one slightly above 5 ppm and the other just below 8 ppm, show motional narrowing over the 25–45 °C temperature range. While it is likely that these same resonances are in slow exchange at lower temperatures, the quality and complexity of the spectra preclude firm conclusions.

Assignment of Ring Flip Partners. Assignment of the resonances to specific rings is necessary before ring flip rates can be estimated by spectral simulations. For a tyrosine ring held rigidly by the surrounding protein, the 3 and 5 ring protons have distinct environments which (usually) results in different chemical shifts. With the exception of accidental degeneracy, the spectrum contains individual resonances of unit intensity for the 3 and 5 protons. Higher temperatures bring about increased internal motion of side chain residues. For tyrosine rings, the dominant motion is a 2-fold flip about the C_β–ring C_1 bond (the χ_2 bond) which interchanges positions of the 3 and 5 protons. For sufficiently fast flips (rates

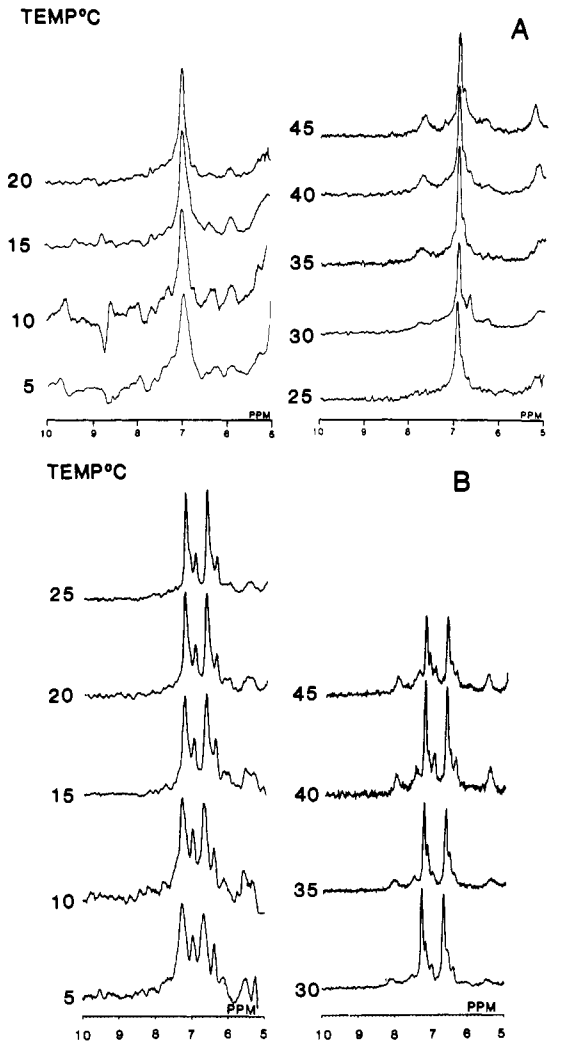

FIGURE 2: Temperature dependence of ^{13}C-decoupled (A) and coupled (B) isotope-edited proton NMR spectra of oxidized [3,5-^{13}C]tyrosine iso-2-cytochrome *c*. Conditions are the same as for Figure 1 except that the iso-2-cytochrome *c* is in the oxidized form.

much greater than the chemical shift differences measured in hertz), the protons see an average environment yielding a single resonance of intensity 2. The averaged chemical shift falls midway between the static or "rigid lattice" chemical shifts for the 3 and 5 protons. Between the two limiting cases, the line shapes are very sensitive to temperature (i.e., changes in flip rates), and comparison with simulated spectra allows estimation of flipping rates. While this is straightforward for resolved resonances from a single tyrosine ring, in the present case there are five tyrosine rings with largely overlapping resonances, and unambiguous assignment of ring flip partners requires additional experiments. We have not been able to solve this problem for the spectra of oxidized cytochrome *c* but summarize a solution for the (decoupled) reduced protein (see Figure 3). Of the five tyrosine rings (denoted I–V), rings I, III, and V are assumed to be in slow exchange at 20 °C and give rise to two resonances each. At 50 °C, the same three rings are in fast exchange and lead to a total of three "averaged" resonances. The remaining two rings (II and IV) give rise to one resonance each over the whole temperature range. Nothing can be said with certainty about the motion of rings II and IV. One or both could be averaged resonances which flip rapidly over the experimentally accessible temperature range. On the other hand, one or both may be instances of chemical shift degeneracy and flip slowly or not at all.

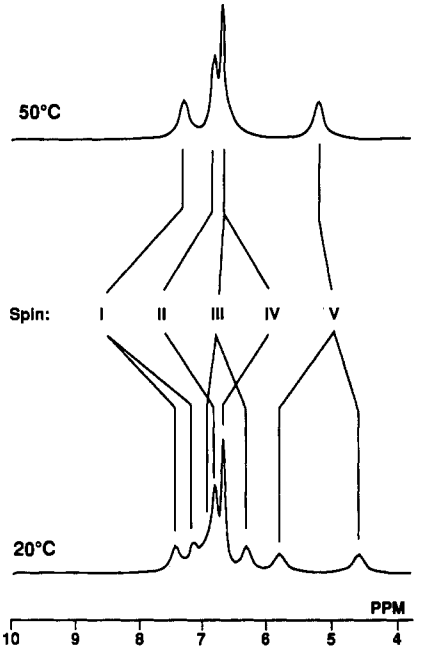

FIGURE 3: Assignment scheme for ring flip partners in reduced iso-2-cytochrome *c* which is used for spectral simulations (Figure 6A,B). Simulated ^{13}C-decoupled isotope-edited spectra are shown for 50 and 20 °C. At 50 °C, all five tyrosine rings are in fast exchange and the 3,5 protons from each ring average to a single resonance of intensity 2. Iso-2 contains five tyrosines, but only four resonance lines are resolved since spin pairs III and IV (near 6.7 ppm) have similar chemical shifts. At 20 °C, three of the five rings (spin pairs I, III, and V) pass over to slow exchange. Each high-temperature resonance (intensity 2) breaks down into a pair of resonances (intensity 1). Two of the tyrosines (spin pairs II and IV) have one resonance each (intensity 2) at both 50 °C and 20 °C. These spin pairs either are in fast (ring flip) exchange throughout the entire temperature range or have 3,5 ring protons with degenerate chemical shifts.

The interpretation outlined in Figure 3 is not without ambiguity. Comparison of simulated (Figure 3) and observed (Figure 1A) spectra shows that in both the fast and slow exchange limits the assignment of resonances to ring I is straightforward since resonances from this ring are almost completely resolved over the entire temperature range. For rings III and V, however, only one of two ring flip related resonances can be observed directly. This is because of severe resonance overlap in the 6.5–7.0 ppm region and spectral distortion from the residual HOD resonance near 4.7 ppm. Thus, the locations for the ring flip related resonances for rings III and V have been assumed and must be verified. The fact that the "averaged" resonance position for ring V is fully resolved at high temperature helps somewhat in locating the corresponding (low temperature) pair of resonances, but even this does not completely solve the problem.

Fortunately, saturation-transfer NOE difference spectroscopy can be used to verify the scheme of Figure 3. The appropriate analogue of this experiment for isotope-edited spectra has been described (Weiss et al., 1986) and can be used to cross-assign 2-fold flip-related resonances. The scheme, outlined in Figure 4, involves taking a difference of proton NOE difference spectra in which a (^{13}C-coupled) tyrosine ring proton resonance is preirradiated (on- or off-resonance) and proton signals are collected with and without broad-band ^{13}C decoupling (Figure 4A). In all cases (Figure 4A, A–D), ^{13}C preirradiation is applied to collapse the J_{CH} doublet so that the ^{13}C-coupled proton can be selectively preirradiated. For ring flip mediated cross-saturation, the net result is a double difference spectrum dominated by two signals (Figure 4B). One is at the frequency of the preirradiated proton, and the

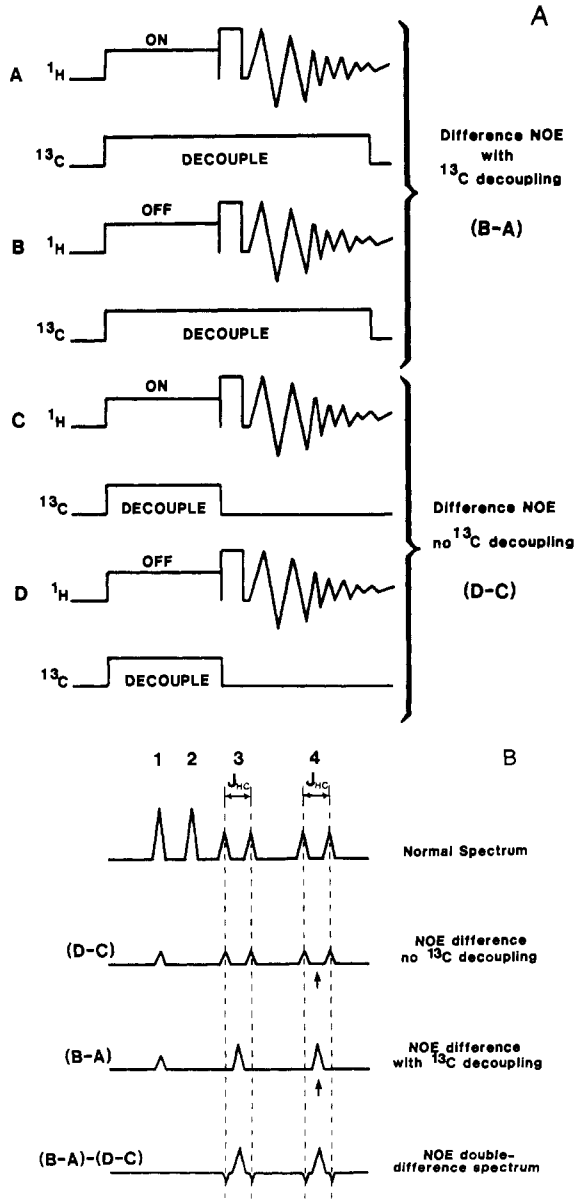

FIGURE 4: (A) Irradiation scheme for isotope-detected NOE double difference spectroscopy. This scheme, similar to that described by Weiss et al. (1986), is used to assign ring flip partners for spin pair III (Figure 5). The scheme used here includes ^{13}C preirradiation to collapse the J_{CH}-coupled proton resonance to allow selective (proton) preirradiation. For our application, the preirradiated and the detected proton resonances are from ^{13}C atoms on opposite sides of the same tyrosine ring (3 and 5 positions). Cross-saturation occurs via chemical exchange (ring flips) rather than by dipolar NOE. (B) Schematic spectra showing the results of the preirradiation scheme for two J_{CH}-coupled resonances related by 2-fold ring flips. Protons within NOE distance of the preirradiated proton resonance but not J_{CH} coupled (1) and protons outside NOE distance (2) are edited from the resulting spectrum. Cross-saturation can occur by both dipolar NOE and chemical exchange. However, ring flip mediated exchange is expected to dominate in the present case.

second response, which is of approximately equal intensity, is at the resonance frequency of the 2-fold flip-related proton. Figure 5 shows the results of this experiment for iso-2-cytochrome *c*. Preirradiation at 6.35 ppm identifies a flip–related resonance at 6.90 ppm, verifying the assignment of flip-related partners for ring III (Figure 3). A similar experiment for ring V gives ambiguous results probably because the expected response at 4.6 ppm coincides with the residual HOD resonance. Regardless, when taken together with the motionally averaged resonance position for the 3,5 protons of ring V at high temperature (Figures 3 and 1), the result in Figure 5 is sufficient

Table I: Parameters for Spectral Simulations[a]

spin pair[b]	ν_0 (ppm)[c]	$\delta\nu_0$ (Hz)[c]	$\Delta\nu_{1/2}$ (Hz)[c]
I	7.31	81	43
II	6.83	0	28
III	6.64	169	43
IV	6.70	0	15
V	5.19	338	43

[a] These parameters taken from the high- and low-temperature extremes of isotope-edited spectra (Figure 1A,B) are used to simulate decoupled (Figure 6A) and coupled spectra (Figure 6B). J_{CH} = 157 Hz is used in simulations of coupled spectra (see Materials and Methods, Data analysis). [b] Each spin pair is composed of a proton at the 3 and 5 positions on the same tyrosine ring. In the absence of J_{CH} coupling, a spin pair gives two resonance lines of unit intensity or a single resonance line of intensity 2, depending on the ring flip rate and the (rigid lattice) chemical shift difference between the 3 and 5 protons ($\delta\nu_0$). In the presence of J_{CH} coupling, each line is split into two lines separated by J_{CH} = 157 Hz. Tentative sequence-specific assignments for each spin pair are given in Table III. [c] ν_0 is the resonance frequency of the 3,5 ring protons in the fast exchange limit where a single resonance line is observed (intensity 2). $\delta\nu_0$ is the difference between the resonance frequencies of the 3 and 5 ring protons in the slow exchange limit where two resonance lines are observed (each of intensity 1). $\Delta\nu_{1/2}$ is the residual line width at half-height of the 3 (or 5) ring proton resonance in the absence of exchange. The frequency parameters are related to the corresponding angular frequency parameters (see Materials and Methods, Data Analysis) by a factor of 2π (for example, $\delta\nu_0 = \delta\omega_0/2\pi$). The simple model used to simulate the spectra assumes that all parameters other than the flip rate, k, are independent of temperature.

Table II: Thermodynamic Reaction Rate Parameters for Ring Flips in Iso-2-cytochrome *c*[a]

spin pair	k (s^{-1}), 20 °C	$\Delta G^{\ddagger}$ (kcal/mol), 20 °C	$\Delta H^{\ddagger}$ (kcal/mol)	$\Delta S^{\ddagger}$ [cal/(mol·K)]
I (Y48)	12	16	28	42
III (Y46)	7	16	28	41
V (Y67)	64	15	36	72

[a] The reaction rate parameters are obtained from least-squares fits to Eyring plots of the temperature dependence of the ring flip rates (Figure 7). Tentative sequence-specific assignments for the spin pairs are given in parentheses and in Table III.

to verify the scheme in Figure 3.

Spectral Simulations and Flip Rate Estimates. To simulate the line-shape changes for the 3,5 tyrosine ring protons in iso-2, we have used procedures described under Materials and Methods together with the parameters given in Table I. Equal intensities have been assumed for each proton resonance to minimize the number of parameters used in simulating the spectra, even though experimental deficiencies in collecting the spectra (e.g., recycle times, decoupling efficiencies) may have distorted relative line intensities somewhat. The least well-defined parameter for the simulations is the line width, $\Delta\nu_{1/2}$. In addition to transverse relaxation, several factors not accounted for explicitly contribute to $\Delta\nu_{1/2}$. These include J_{ortho} = 8.5 Hz splitting of the 3,5 proton resonance lines by the 2,6 protons, line intensity differences, and motions other than 2-fold ring flips. Regardless, results of the simulations for decoupled (Figure 6A) and coupled (Figure 6B) spectra compare well with observed spectra (Figure 1A,B).

Ring Flip Energetics. Reaction rate theory (Glasstone et al., 1941) is used to evaluate enthalpy and entropy differences between the ground state and transition state for ring flips. Eyring plots (Figure 7) show that two of the rings (I and III) have essentially the same activation parameters while ring V has a larger enthalpy and entropy of activation. The results are summarized in Table II where activation enthalpies and entropies are compared for the three rings. Also included is

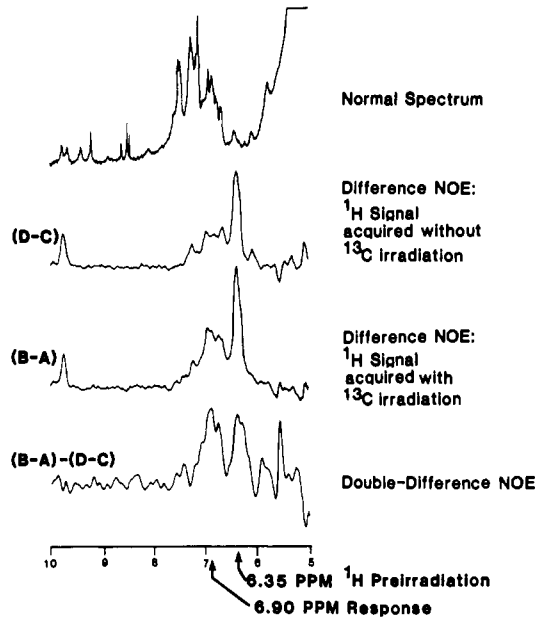

FIGURE 5: Assignment of ring flip partners in [3,5-^{13}C]tyrosine iso-2-cytochrome *c* with isotope-detected double difference spectroscopy (see Figure 4 for irradiation scheme). Proton preirradiation at 6.35 ppm is applied to a (3 position) tyrosine ring proton resonance which is resolved in the isotope-edited NOE difference spectrum (Figures 1A and 3). NOE difference spectra are acquired without ^{13}C decoupling (D – C) and with ^{13}C decoupling (B – A). In the double difference, (B – A) – (D – C), a response near 6.90 ppm identifies the resonance of the 2-fold flip-related (5 position) tyrosine ring proton. The resonance slightly below 10 ppm in the intermediate difference spectra, (D – C) and (B – A) (edited out of the final double difference spectrum), is from the heme meso βH. Spectra are obtained at 20 °C in 99.7% deuterium oxide/0.1 M sodium phosphate, pD 6.0, with cytochrome *c* concentrations of 0.5–0.8 mM. Samples were reduced by bubbling with nitrogen gas and adding a small amount of solid sodium dithionite.

a comparison of rates and activation free energies at 20 °C.

Discussion

Measurement of aromatic ring motion has remained experimentally challenging, despite its importance as a probe of protein internal dynamics. Most NMR techniques for investigation of motion require highly resolved spectra. It is necessary to monitor not only resonance position but also subtle changes in line shape over a wide temperature range. As a result, quantitative investigation of ring motion is limited to proteins of the lowest possible molecular weight (Snyder et al., 1975; Wagner et al., 1976) or, as in the present case, requires introduction of specific isotopic labels (Hull & Sykes, 1975). Both approaches have drawbacks. Small proteins exhibit many but perhaps not all of the dynamic features to be found in larger proteins. Introduction of isotopic labels may in some instances perturb the system under study (e.g., ^{19}F) or requires complex spectroscopic schemes that can distort line intensities (e.g., isotope-edited spectroscopy). Regardless, results presented here show that isotope-edited NMR spectroscopy can be valuable for studies of aromatic ring dynamics for small- to medium-sized proteins.

Spectral Assignments for 3,5 Tyrosine Ring Protons in Reduced Iso-2. An understanding or ring flip rates and energetics in terms of the iso-2 structure (Murphy and Brayer, unpublished results) (Leung et al., 1989) must be based on firm sequence-specific resonance assignments for the tyrosine ring protons. These are not yet available. Nevertheless, the assigned resonances for the 3,5 tyrosine ring protons in horse cytochrome *c* can be compared with the chemical shifts for unassigned 3,5 tyrosine ring proton resonances in iso-2 (Table

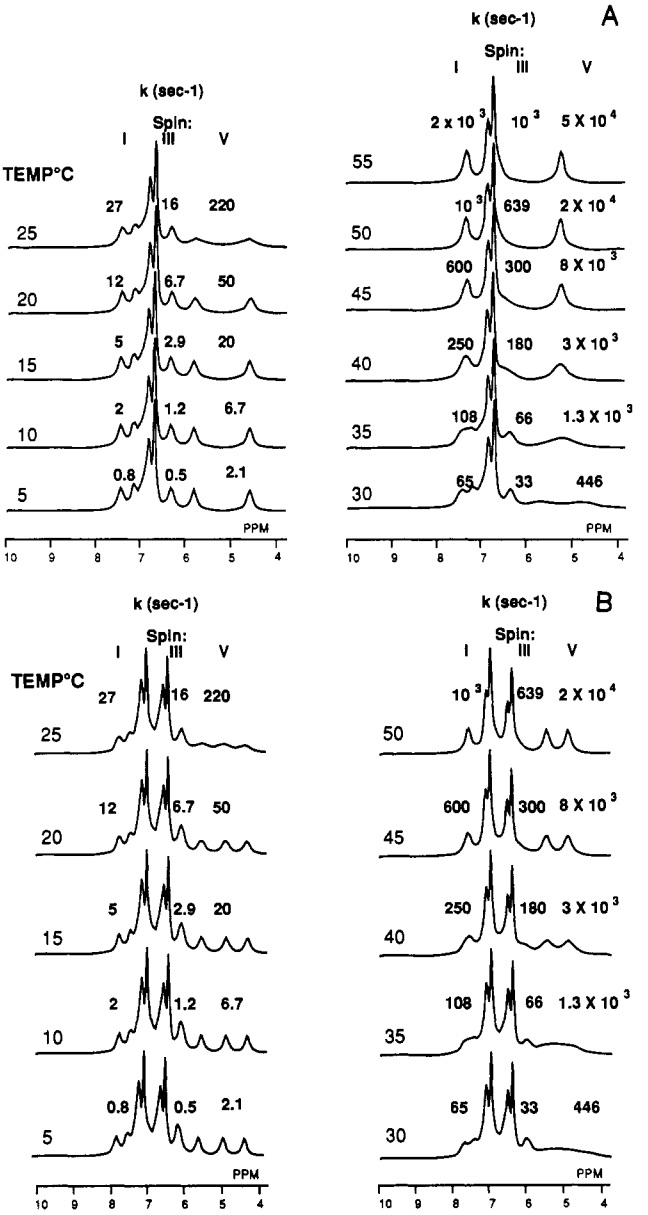

FIGURE 6: Spectral simulations of the temperature dependence of the isotope-edited NMR spectra of reduced [3,5-^{13}C]tyrosine iso-2-cytochrome *c*. (A) J_{CH}-decoupled and (B) J_{CH}-coupled spectra are shown. Ring flip rates, *k*, are indicated for the three tyrosines (spin pairs I, III, and V) which pass from slow exchange to fast exchange as temperature is increased. Simulations are carried out as described under Data Analysis in Materials and Methods using parameters given in Table I

III). The comparison suggests tentative assignments for iso-2 which must eventually be verified by more rigorous methods. The correlation is striking. With the exception of Tyr-97,[2] our suggested resonance assignments for iso-2 have chemical shifts within 0.17 ppm of the corresponding assigned resonances in horse cytochrome *c*. So why is there not better agreement between the chemical shifts for Tyr-97 in horse cytochrome *c* and iso-2? One possibility is that the proposed horse cytochrome *c* assignments (Eley et al., 1982; Wand et

[2] The vertebrate cytochrome *c* numbering system is used to denote amino acid positions in order to facilitate comparison between members of the cytochrome *c* family. Iso-2 has nine additional amino-terminal residues and one residue less on the carboxy terminus compared to vertebrate cytochromes *c*. Thus, the vertebrate numbering of iso-2 starts at position −9 and extends to position 103 [see Dickerson (1972) and Hampsey et al. (1986)]. For example, Tyr-67 in the vertebrate numbering system corresponds to Tyr-76 in the iso-2 numbering system.

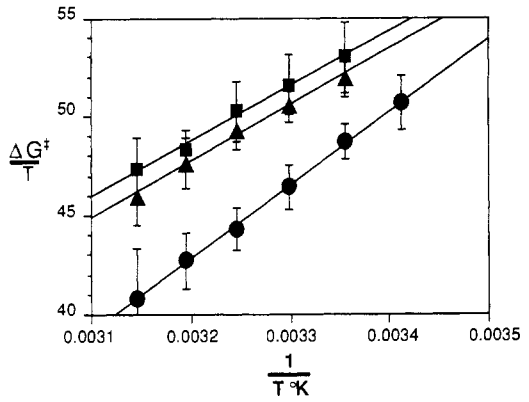

FIGURE 7: Eyring plots of tyrosine ring flip rates, k, for spin pairs I (▲), III (■), and V (●) in reduced iso-2-cytochrome c. The rates, k, are estimated from spectral simulations (Figure 6). Rates are plotted only for the temperature range where the line shape is a strong function of temperature: 25–45 °C for spin pairs I and III; 20–45 °C for spin pair V. The solid lines are least-squares fits to the flip rates and yield the thermodynamic reaction rate parameters given in Table II. Error bars, like flip rates, are estimated by spectral simulation.

al., 1989) for Tyr-97 are in error in that the 3,5 and 2,6 proton resonance assignments need to be switched. If this is the case, the 3,5 proton chemical shifts for Tyr-97 in iso-2 and horse cytochrome c then agree to within 0.02 ppm (Table III). An assignment error of this nature is understandable for horse cytochrome c where the distinction between the 2,6 and 3,5 protons is based on NOE intensities rather than, as in iso-2, selective isotopic labeling. Additional support for the proposed assignment scheme is available for Tyr-46 and Tyr-48, which, according to the 1.9-Å X-ray structure (Murphy and Brayer, unpublished results) have a 5 to 3 position ring proton interresidue distance of 4.0 Å. A resolved but weak NOE is observed between resonances at 6.94 ppm (Tyr-46, ring 5H) and 7.14 ppm (Tyr-48, ring 3H), which may be the expected response (Nall, Feng, Englander, and Roder, unpublished results). All other interresidue distances between 5 and 3 position ring protons on Tyr-46 and Tyr-48 are in excess of 5 Å.

Tyrosine-67 appears to have been difficult to assign in horse cytochrome c. Wand et al. (1989), in the most complete list of assignments for horse cytochrome c to date, do not give values for the Tyr-67 ring protons. Eley et al. (1982) provide assignments for the Tyr-67 ring, but only at relatively high temperatures (57–93 °C), and no distinction is made between 2,6 and 3,5 ring protons. The results with iso-2 suggest reasons for the difficulty. At low temperatures, the chemical shift (in iso-2) for the Tyr-67 ring 5 proton (4.56 ppm, Table III) is close to the residual HOD resonance (4.71 ppm) and will be bleached from the spectrum by HOD presaturating pulses. In addition, the ring flip rates for Tyr-67 at low temperatures are such that the ring 3 proton (5.82 ppm) will be bleached from the spectrum by (chemical exchange mediated) cross-saturation from the ring 5 proton. At high temperatures, the (exchange averaged) chemical shift for the 3,5 ring protons (5.19 ppm) is likely to be reduced in intensity by HOD presaturation. In this regard, isotope-edited NMR spectroscopy has a considerable advantage for observing protein resonances near the HOD resonance. The difference NOE methods used in (isotope) editing spectra provide good water suppression without HOD presaturating pulses, at least in deuterium oxide solutions.

Assignments of a different sort, cross-assignments of resonance pairs in slow exchange, provide a basis for spectral simulations and flip rate estimates. Only three of the five

Table III: Tentative Sequence-Specific Assignments for Reduced Iso-2: Comparison to Horse Cytochrome c[a]

assignment	horse cytochrome c	yeast iso-2-cytochrome c
Y46[b]	(corrected values[b])	(spin pair III)
ring 3H	6.41 [6.42]	6.33
ring 5H	6.99 [7.03]	6.95
ring 3,5H[c]	{6.70, [6.73]}	6.64
Y48		(spin pair I)
ring 3H	7.09	7.16
ring 5H	7.29	7.46
ring 3,5H[c]	{7.19}	7.31
Y67		(spin pair V)
ring 3H		5.82
ring 5H		4.56
ring 3,5H[c]	[5.13 or 6.73][d]	5.19
Y74		(spin pair IV)
ring 3H		
ring 5H		
ring 3,5H[c]	6.60 [6.61–6.64]	6.70
Y97		(spin pair II)
ring 2H	6.54	
ring 6H	7.08	
ring 2,6H[c]	{6.81}	
ring 3H	5.46	
ring 5H	6.67	
ring 3.5H[c]	{6.07}	6.83

[a] Iso-2-cytochrome c assignments are based on comparison to chemical shifts of assigned tyrosine protons for horse cytochrome c. These two cytochromes c have 54% sequence identity. Most amino acid differences occur on the surface with the hydrophobic core and the aromatic residues largely conserved. The chemical shifts given for horse cytochrome c are from Wand et al. (1989) or, in square brackets, from Eley et al. (1982). Chemical shifts given for iso-2 are the values used in the simulations of the isotope-edited spectra. [b] Horse cytochrome c has F46 in place of the Y46 found in iso-2. The chemical shifts for 3 and 5 ring protons of a tyrosine at position 46 (in horse cytochrome c) have been estimated by subtracting 0.5 ppm from the chemical shifts of F46. This corrects for conformation-independent shift differences between the 3,5 protons of tyrosine and phenylalanine (Wuthrich, 1976). [c] This gives the chemical shift of the 3,5 ring protons when a single resonance is observed for the spin pair. This occurs when the tyrosine ring is in fast chemical exchange or when the (rigid lattice) shift differences are unresolved. If chemical shifts in the fast exchange limit have not been reported explicitly, then averages of the (rigid lattice) chemical shifts are indicated in braces. [d] These assignments from Eley et al. (1982) are at relatively high temperature (57–93 °C) and do not distinguish between the 3,5 and the 2,6 ring proton resonances for Tyr-67.

tyrosines exhibit slow exchange, so only three slowly exchanging resonance pairs need to be cross-assigned. Spin pair I is easily assigned since its resonances are resolved in slow, intermediate, and fast exchange. Spin pair III is assigned on the basis of the isotope-detected double difference spectrum (Figure 5). In the fast exchange limit, the 3,5 proton shifts of spin pair III should average to about 6.64 ppm and overlap with the resonance of spin pair IV (6.70 ppm). The fact that (at high temperatures) the resonance line at 6.64–6.7 ppm is from two tyrosines (spin pairs III and IV) is supported by the approximately double line intensity and by splitting of this resonance line at 60 °C (data not shown). For spin pair V, only one of the two partners is observed at low temperature (the ring 3 proton at 5.82 ppm). At high temperature, this resonance averages with its partner to give the 3,5 ring proton resonance at 5.19 ppm, fixing the location of the (low temperature) 5 ring proton resonance at 4.56 ppm. Having located the expected position for the (low temperature) 5 ring proton resonance (spin pair V), it is clear why this resonance is not observed directly: it coincides almost exactly with the residual HOD resonance.

Ring Flips in Other Proteins. Aromatic ring flip motion in basic pancreatic trypsin inhibitor (BPTI) is better char-

acterized than for any other protein. Two of the four tyrosines and one of four phenylalanines are in slow chemical exchange at low temperature and fast exchange at high temperatures (Wagner, 1987; Wagner et al., 1976). The remaining tyrosines and phenylalanines are in fast or intermediate exchange over the accessible temperature range. Flip rates at low temperatures (4 °C) are in the range of 1–33 s^{-1}, rising to about 50000 s^{-1} at high temperature (80 °C). Activation enthalpies and entropies vary dramatically from ring to ring within a range of $\Delta H^{\ddagger}$ = 17–37 kcal/mol and $\Delta S^{\ddagger}$ = 11–35 cal/(mol·K). In further work, the pressure dependence of the flip rates was used to determine activation volumes for two of the of the rings, $\Delta V^{\ddagger} = 50 \pm 10$ Å^3 (Phe-45) and $\Delta V^{\ddagger} = 60 \pm 20$ Å^3 (Tyr-35) (Wagner, 1980). There was little change in aromatic ring flip rates in chemically modified forms of BPTI, except in the immediate vicinity of the chemical modification (Wagner et al., 1979). The flip rates, activation enthalpies, and activation entropies for iso-2 are of the same order of magnitude as for BPTI (Table II).

Saturation-transfer NMR spectroscopy has been used to measure the flip rate and activation parameters for a single tyrosine in horse cytochrome *c* (Campbell et al., 1976). Although the residue was not identified in the original report, later work indicates that it was Tyr-97 (Eley et al., 1982). Spectral simulations give a flip rate of 8 s^{-1} (25 °C) and $\Delta H^{\ddagger}$ = 23 kcal/mol, $\Delta S^{\ddagger}$ = 23 cal/(mol·K) (Campbell et al., 1976). For iso-2, Tyr-97 is always in fast exchange or, alternatively, the 3 and 5 ring proton resonances are degenerate.

Ring Flip Motion and Structure in Reduced Iso-2. Discussion of ring motion in iso-2 will assume the validity of the (tentative) assignments (Table III). In terms of which tyrosines flip slowly at low temperatures, there is good agreement between horse cytochrome *c* and yeast iso-2. A possible exception is Tyr-97 which may be in fast exchange at low temperatures in iso-2 but in slow exchange (below about 37 °C) in horse cytochrome *c*. For aromatic rings corresponding to tyrosines-46, -48, and -67 in iso-2, those at positions 46 and 48 are in slow exchange at low temperature and fast exchange at high temperature in both proteins. Tyr-67, which is in slow exchange at low temperature in iso-2, is probably in slow exchange in horse cytochrome *c* too, judging from the difficulty in assigning this residue at low temperatures (Eley et al., 1982; Wand et al., 1989). It is especially interesting to note that Tyr-46 and Tyr-48, which make up a closely interacting pair of residues in cytochrome *c*, have the same ring flip activation parameters. This suggests the possibility of correlated flipping motions for these two side chains where similar local environments and breathing motions allow flips for both residues.

Comparison with X-ray Structure. Murphy and Brayer have recently solved the 1.9-Å resolution X-ray structure for reduced iso-2-cytochrome *c* with an *R* factor of 18.9% (Murphy and Brayer, unpublished results). It is interesting that the tyrosines which are in slow ring flip exchange at low temperatures (Tyr-46, Tyr-48, and Tyr-67) are those that have the smallest thermal *B* factors (Murphy and Brayer, unpublished results). For example, tyrosines-46, -48, and -67 have *B* factors that range from 5 to 14 Å^2 while *B* factors for tyrosines-74 and -97 are 14–27 Å^2. While this suggests a correlation between *B* factors and ring flip motion, it is important to keep in mind that the flip rates for tyrosines-74 and -97 are undetermined: the static chemical shifts for these residues are unknown or degenerate. Moreover, *B* factors, which measure a combination of local motion and disorder, might be better indicators of the appropriateness of the two-site exchange model than of ring flip motion itself. Regardless, the fact that the tyrosines in slow exchange at low temperatures have smaller *B* factors suggests a possible relationship between the energetically accessible ground states of a residue and activation free energy barriers for ring flips.

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Registry No. L-Tyr, 60-18-4; cytochrome *c*, 9007-43-6.

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Extremely Thermostable D-Glyceraldehyde-3-phosphate Dehydrogenase from the Eubacterium *Thermotoga maritima*†

Alexander Wrba, Anita Schweiger, Verena Schultes, and Rainer Jaenicke*
Institut für Biophysik und Physikalische Biochemie der Universität Regensburg, D-8400 Regensburg, FRG
Péter Závodszky
Institute of Enzymology, Biological Research Centre, Hungarian Academy of Sciences, Budapest, Hungary
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ABSTRACT: D-Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from *Thermotoga maritima*, a hyperthermophilic eubacterium, has been isolated in pure crystalline form. The enzyme is a homotetramer with a subunit molecular mass of 37 kDa. The sedimentation coefficient of the native enzyme is 7.3×10^{-13} s, the isoelectric point is 4.6, and the specific absorption coefficient $A_{280nm}^{1\%,\,1\,cm} = 8.4$. The enzyme shows extreme thermal stability: differential scanning calorimetry yields a transition temperature (T_m) of 109 °C for the NAD-saturated enzyme. Thermal deactivation occurs at $T > 90$ °C. The physicochemical characteristics of the enzyme suggest that its gross structure must be very similar to the structure of GAPDHs from mesophilic sources. The amino acid composition does not confirm the known "traffic rules" of thermal adaptation, apart from the Lys → Arg exchange. One reactive and at least two buried SH groups can be titrated with 5,5′-dithiobis(2-nitrobenzoate). The highly reactive SH group is probably the active-site cysteine residue common to all known GAPDHs. The activation energy of the glyceraldehyde 3-phosphate oxidation reaction decreases with increasing temperature. This functional behavior can be correlated with the temperature-dependent changes of both the intrinsic fluorescence and the near-UV circular dichroism; both indicate a temperature-dependent structural reorganization of the enzyme. Hydrogen–deuterium exchange reveals significantly increased rigidity of the thermophilic enzyme if compared to mesophilic GAPDHs at 25 °C, thus indicating that the conformational flexibility is similar at the corresponding physiological temperatures. The increase in $\overline{\Delta G}$, i.e., the Gibbs energy of the average microunfolding exposing peptide hydrogens to the solvent, is 5.2 kJ/mol, going from the mesophilic to the thermophilic enzyme. The effect may be attributed to the increased saturation of the structure with nonpolar contacts.

Glyceraldehyde-3-phosphate dehydrogenases (GAPDHs,[1] EC 1.2.1.12) have been isolated from a variety of species (Harris & Waters, 1976), including mesophilic (Krebs et al., 1953; d'Alessio & Josse, 1971; Misset et al., 1987), moderately thermophilic (Amelunxen, 1966; Hocking & Harris, 1973; Fujita et al., 1976; Crabb et al., 1977), and (hyper)thermophilic microorganisms (Hensel et al., 1987; Fabry & Hensel, 1987). The latter enzymes show increased thermal stability and are enzymatically active at elevated temperatures.

We observed a high level of GAPDH activity in the cell homogenate of *Thermotoga maritima*. This microorganism is an extremely thermophilic eubacterium, isolated from geothermally heated locales on the sea floor (Huber et al., 1986). It grows optimally between 80 and 90 °C. Our preliminary studies indicated that the enzyme is remarkably stable, even at 100 °C, thus representing the most stable glycolytic enzyme isolated so far. This extreme thermostability opens a way to study enzyme action and regulation, as well as protein folding in an extended temperature range.

A series of homologous GAPDHs from various sources, with widely differing thermal stabilities, have been shown to be

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* Address correspondence to this author at Biochemie II, Universität Regensburg, Universitätsstrasse 31, D-8400 Regensburg, FRG.

[1] Abbreviations: GAPDH, D-glyceraldehyde-3-phosphate dehydrogenase; EDTA, ethylenediaminetetraacetic acid; TEA, triethanolamine; CD, circular dichroism; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTE, dithioerythritol; PEG, poly(ethylene glycol); DSC, differential scanning calorimetry.

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