Kinetic Studies on the Hydrazine and Phenylhydrazine Reductions of the *Escherichia coli* R2 Subunit of Ribonucleotide Reductase

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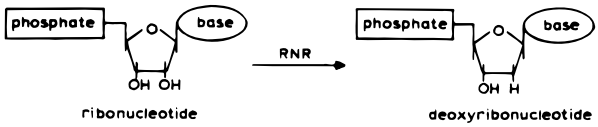
Received November 22, 1995[⊗]

Samples of the *Escherichia coli* R2 protein of ribonucleotide reductase (RNR) normally have two components, the fully active tyrosyl radical ($Tyr^{\bullet}$) and Fe^{III}_2-containing protein (∼60%) and the Fe^{III}_2-only met-R2 form (∼40%). Reaction with the 1- or multi- (maximum 4-) equiv reagent hydrazine under anaerobic conditions gives biphasic kinetics. From UV−vis absorbance changes, the first stage of reaction corresponds unexpectedly to reduction of the Fe^{III}_2 met-R2 component, rate constant 7.4×10^{-3} M^{-1} s^{-1} (25 °C) at pH 7.5. The slower second stage is assigned as net reduction of $Tyr^{\bullet}$ and one Fe^{III} of the Fe^{III}_2 center, rate constant 1.7×10^{-3} M^{-1} s^{-1}. Separate experiments with met-R2 protein, and previous evidence from EPR spectroscopy for the formation of an $Fe^{II}Fe^{III}$ intermediate, support such a mechanism. Reduction of the second Fe^{III} is then rapid. The corresponding reduction of the Tyr122Phe R2 variant gives a rate constant of 7.7×10^{-4} M^{-1} s^{-1}, which is substantially (×10) less than that for met-R2. This is in part explained by the decreased reduction potential of the variant. From pH variations in the range pH 6.6−8.5, N_2H_4 is the prime reactant with little or no contribution from $N_2H_5^+$ (pK_a 8.2). Phenylhydrazine (250 mV) is unable to reduce the Fe^{III}_2 center, and reacts only with the tyrosyl radical (a 1-equiv process) of the active R2 protein (0.184 M^{-1} s^{-1}). The reaction is $>10^2$ times faster than the 2-equiv N_2H_4 reduction of $Tyr^{\bullet}$ and Fe^{III}_2. The pK_a for $C_6H_5N_2H_4^+$ is 5.27, $C_6H_5N_2H_3$ is the dominant species present under the pH conditions (6.5−8.5) investigated, and no pH dependence is observed. Contrary to a previous report, we conclude that the stability of the diimide (N_2H_2) does not allow separate studies of the reduction of the R2 protein with this reagent.

Introduction

The enzyme ribonucleotide reductase (RNR) catalyzes the formation of 2′-deoxyribonucleotides from the four different ribonucleoside diphosphates, a reaction essential to DNA synthesis (eq 1).[1] The need for all living organisms to have



(1)

this capability emphasizes the important key role of RNR in biology. The active enzyme consists of two subunits, here referred to as R1 and R2, in 1:1 amounts. Both are homodimers of molecular mass 2 × 85.5 and 2 × 43.5 kDa, respectively, for the *Escherichia coli* protein.[2] In the case of R1, two cysteines (Cys-225 and Cys-462) are believed to provide the reducing equivalents for nucleotide reduction by electron- and proton-transfer reactions.[3] A third cysteine Cys-439 is first oxidized to a thiyl ($RS^{\bullet}$) radical, which initiates nucleotide reduction by 3′-hydrogen atom abstractions from the nucleotide substrate in (1).[4] The active form of R2 contains a tyrosyl radical ($Tyr^{\bullet}$), generated from the deprotonated phenolate form, which is stabilized by magnetic coupling to the nearby Fe^{III}_2.[5,6] The function of the $Tyr^{\bullet}$ proposed is to generate the thiyl radical by long-range-coupled electron and proton transfers to R1. As yet, 100% formation of $Tyr^{\bullet}$ has not been observed, and the maximum content of $Tyr^{\bullet}$ per dimer R2 so far reported is 1.5.[7] The value normally found is around 1.2 with only 60% of the wild-type protein containing $Tyr^{\bullet}$ and the rest present as tyrosine (here referred to as TyrH). The Fe content on the other hand is reported to be close to the 4.0 mol/mol of dimer upper limit, with adventitiously bound mononuclear Fe^{III} at a level <0.1 mol/mol of R2.[8] The two Fe^{III}'s are μ-oxo bridged and antiferromagnetically coupled with $-J = 87$ cm^{-1}.[9] X-ray crystal structures of inactive met-R2 protein,[10,11] and of the fully reduced Fe^{II}_2 protein[12] have been reported. In met-R2 the bonding is as illustrated, with the Tyr-122 close to but not coordinated to the Fe^{III}_2 site (the phenolate O atom is 5.3 Å from Fe_a). Active enzyme can be regenerated by the reaction of the fully reduced protein with O_2, which coordinates to Fe^{II}_2 and oxidizes R2 to the active $Tyr^{\bullet}$ and Fe^{III}_2 form.[13,14] As well

[⊗] Abstract published in *Advance ACS Abstracts,* July 1, 1996.

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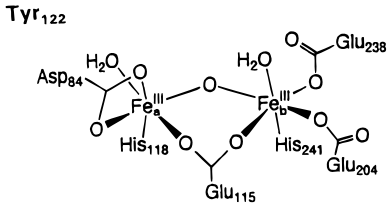
S0020-1669(95)01501-1 CCC: $12.00



as stabilizing the tyrosyl radical, this therefore represents a further role for the binuclear Fe site.

Kinetic studies on the reduction of the Tyr• radical of active R2 by hydroxyurea and a number of hydroxamic acid derivatives, $Tyr^{\bullet} + H^{+} + e^{-} \rightarrow TyrH$ have been reported.[15] With dithionite and methyl viologen as a mediator reduction of Tyr• and then $Fe^{III}_2 \rightarrow Fe^{II}_2$ has been reported.[16] Other reagents which have been studied include 10 different hydrazines and a similar number of hydroxylamines.[17] However half-lives only were considered in the latter study, and selected reactions clearly merit more detailed investigation. Accordingly we report here studies on the reactions of R2 with hydrazine and phenylhydrazine, including the determination of rate laws, rate constants, and effects of pH. Particularly important is the reaction with hydrazine, which is shown to be a rather unusual biphasic process. Data for the reduction of met-R2 and the Tyr122Phe variant are also included.

Experimental Section

Protein. Wild-type *E. coli* R2 and mutant Tyr122Phe R2 protein were prepared from *E. coli* overproducing strains,[18] kindly provided by the group of Professor B.-M. Sjöberg, University of Stockholm, Sweden. Concentrated ~0.3 mM solutions of R2 were stored in small batches at −80 °C. Protein was made air-free by dialyzing against deaerated buffer. Met-R2 was prepared by reacting active R2 (~0.5 mM) with hydroxyurea (50 mM) at 25 °C for ~30 min, after which excess hydroxyurea was removed using a Sephadex G-25 column. The diluted met-R2 solution was reconcentrated by dialysis against deaerated buffer of 50 mM Tris/HCl at pH 7.5 containing 20% glycerol.[19] The UV−vis spectrum of the Fe^{III}_2 met-R2 has peaks at 325 and 375 nm similar to those observed for hemerythrin.[20] In the case of active RNR, an additional sharp band at 410 nm ($\epsilon \sim 6600\ M^{-1}\ cm^{-1}$),[21] is assigned to Tyr•, Figure 1. Tyr• also has a weak absorbance at ~600 nm, which contributes to the characteristic green color of active R2. Fully reduced Fe^{II}_2 protein has no absorbance at >300 nm. Concentrations of protein were determined spectrophotometrically using the difference in absorption coefficients $\epsilon_{280} - \epsilon_{310} = 120\,000\ M^{-1}\ cm^{-1}$.[22]

Reductants. Hydroxyurea ($NH_2CONHOH$), crystalline hydrazine dihydrochloride ($N_2H_4 \cdot 2HCl$), crystalline phenylhydrazine hydrochloride ($C_6H_5N_2H_3 \cdot HCl$), all from Sigma Chemical Co., Ltd., and liquid hydrazine hydrate ($N_2H_4 \cdot H_2O$), from Lancaster Synthesis, were used without further purification.

Buffers. The following were used at 50 mM concentrations: tris(hydroxymethyl)aminomethane (Tris; pH range 7.1−8.9), *N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonic acid) (Hepes; pH range 6.8−8.2), and 2-morpholinoethanesulfonic acid (Mes; pH range 5.5−6.7), also from Sigma. The pH was measured before and after kinetic runs on a Radiometer pHM62 pH-meter fitted with a Russell CWR-322 glass electrode.

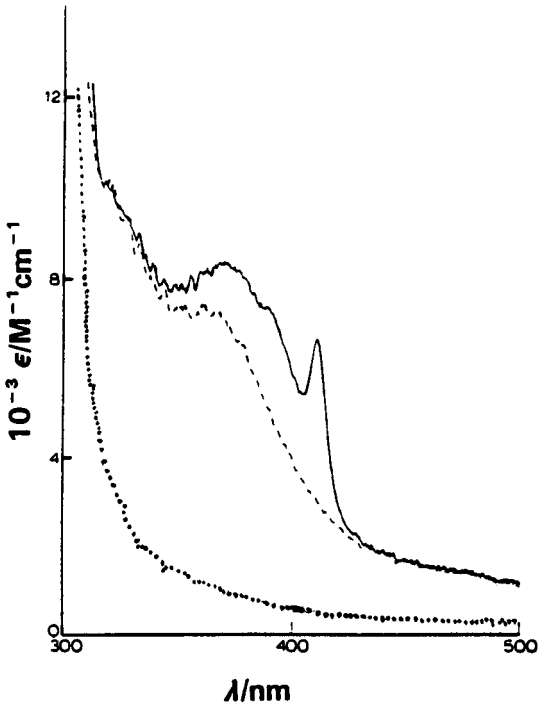


Figure 1. UV−vis spectra of different oxidation states of *E. coli* R2 ribonucleotide reductase in its active Tyr• and Fe^{III}_2 oxidized form (—), the met-R2 form in which the Tyr• but not the Fe^{III}_2 is reduced (- - -), and the fully reduced TyrH and Fe^{II}_2 form (···).

Electrochemistry. A three-electrode system was used, consisting of a Pt-wire auxiliary electrode and an EG & G glassy carbon working electrode against an Ag/AgCl (1 M KCl) reference electrode. Phenylhydrazine hydrochloride did not give reversible cyclic voltammograms, and midpoint redox potentials from square-wave voltammograms (SWV) were obtained giving E_{swv} vs NHE. The value obtained was 250 mV at pH 7.5 (50 mM Tris/HCl), $I = 0.100$ M (NaCl). Reduction potentials for phenylhydrazinesulfonates, using methylene blue as mediator, have been reported to be ~440 mV.[23]

Reaction Products. Hydrazine is known to react in 1- or multi- (maximum 4-) equiv steps, overall equations as in (2) and (3), yielding

$$N_2H_4 \rightarrow {}^1/_2N_2 + NH_3 + H^+ + e^- \quad (2)$$

$$N_2H_4 \rightarrow N_2 + 4H^+ + 4e^- \quad (3)$$

products N_2 and NH_3. In acidic solutions, reduction potentials for (2) and (3) are quoted as −1.74 and +0.23 V, respectively, while in basic solution (3) gives −1.16 V, indicating strong reducing properties.[24,25] Diazene, if formed as an intermediate, is unstable at temperatures above −180 °C.[26] For maximum attainable R2 concentrations (~1 mM), the calculated amount of N_2 released is small and does not enable us to make a distinction between (2) and (3). Arylhydrazines behave as 2-equiv reductants, giving first an azo compound,[27] which is unstable, and the overall reaction can be expressed as in (4).

$$C_6H_5NHNH_2 \rightarrow C_6H_5N{=}NH \rightarrow C_6H_6 + N_2 \quad (4)$$

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Kinetic Studies. Conventional time range ($t_{1/2}$ > 1 min) runs were monitored by UV−vis spectrophotometry with the reductant in >10-fold excess of the protein concentration. All kinetic studies were carried out anaerobically (N_2) at 25.0 ± 0.1 °C and within the pH range 6.5−8.5. The ionic strength was adjusted with NaCl (BDH, AnalaR) to 0.100 ± 0.001 M. All manipulations for kinetic runs were carried out in a Miller-Howe glovebox, O_2 level <2 ppm. Both reductants have protonated forms with pK_a values, (5) and (6), reported as 8.23 and

$$N_2H_5^+ \rightleftarrows N_2H_4 + H^+ \quad (5)$$

$$C_6H_5N_2H_4^+ \rightleftarrows C_6H_5N_2H_3 + H^+ \quad (6)$$

5.27, respectively.[28,29] Allowances were made for any contributions to pH and ionic strengths from these compounds. The Tyr• decay can be monitored at 410 nm (ϵ = 66 00 M^{-1} cm^{-1}) whereas the Fe^{III} decay is dominant at 370 nm (ϵ = 8700 M^{-1} cm^{-1}). For studies on active (wild-type) *E. coli* R2, repeat scans (300−500 nm) were recorded at appropriate time intervals. For met-R2 and Tyr122Phe R2, repeat scan spectra or absorbance changes at a fixed wavelength (370 nm) were used. Kinetic runs were monitored on either a Shimadzu UV-2101PC or a Perkin-Elmer Lambda 9 spectrophotometer.

Reactions which are uniphasic give linear plots of $\ln(A_t - A_\infty)$ against time t, and first-order rate constants (k_{obs}) were obtained from the slopes. For biphasic reactions, such plots yielded a linear portion (slope k_{2obs}) for the latter stages only. To obtain the rate constant for the earlier stages, the intercept at $t = 0$ gives $\ln(x)$ and allows a modified plot of $\ln[A_t - A_\infty - x\exp(-k_{2obs}t)]$ vs t to be carried out, the slope of which yields k_{1obs} for the first phase of reaction.

We also employed a method used by others[30,31] to determine rate constants for the Tyr• decay by measuring the peak height at 410 nm relative to a fitted base-line (FBL) method, illustrated in the inset to Figure 2. This requires a straight line to be drawn between the absorbances at 400 and 420 nm as the reaction progresses and plotting $\ln(\Delta A)$ versus time (rate constant from the slope). The approach was tested with reference to the reaction of hydroxyurea with active R2 in which Tyr• only is reduced. Rate constants (25 °C) determined in the normal manner at 370 and 410 nm give identical values of 0.46 M^{-1} s^{-1}.[15] Using the FBL method, rate constants of 0.55 M^{-1} s^{-1} (20% higher) were obtained. Systematic errors appear to be introduced by the way in which the base line is constructed, resulting in higher rate constants. Nevertheless the method does have value in targeting the Tyr• decay.

Treatment of Data. Unweighted least-squares fits were used to obtain second-order rate constants from the dependence of first-order rate constants on reductant concentrations. A nonlinear least-squares fitting program was used for pH studies to obtain pK_a values.

Results

Hydrazine Reduction of Met-R2. We report the met-R2 reaction first for reasons which will become apparent in the next section. First-order rate constants k_{obs} obtained by monitoring the uniphasic absorbance decay at 370 nm with reductant in >10-fold excess are listed in Table 1. At pH 8.47, k_{obs} shows a linear dependence on the total hydrazine concentration $[N_2H_4]_T$, yielding second-order rate constants k_{Fe}. The variation of k_{Fe} with pH suggests that $N_2H_5^+$ has little or no reactivity as compared to N_2H_4. A full treatment is considered later, (7)−(10). The single rate-determining step is consistent with a two-electron reduction by the hydrazine.

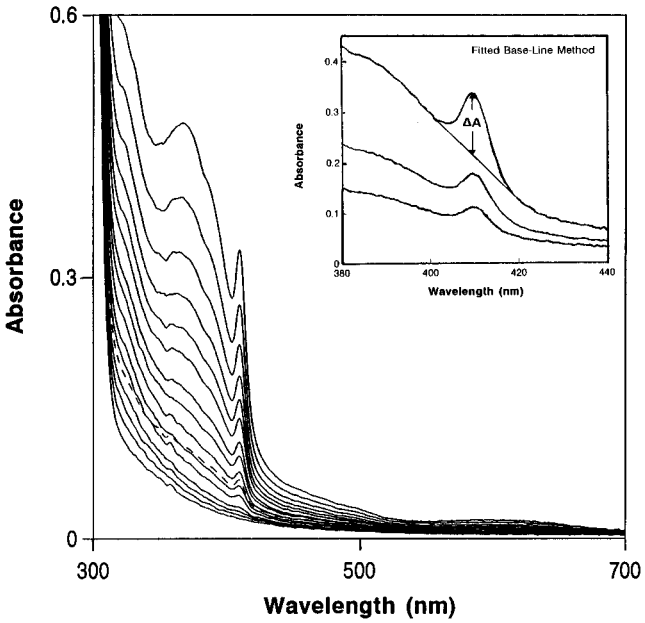

Figure 2. UV−vis scan spectra for the reaction (25 °C) of hydrazine (22 mM) with the active R2 form of ribonucleotide reductase (50 μM) at pH 8.46 (50 mM Tris/HCl), I = 0.100 M (NaCl). The first six spectra were recorded at 600 s, then five at 1000 s, two at 5000 s, and two at 10^4 s intervals. The broken line was obtained after completion of the reaction and admitting air. The inset illustrates the fitted base-line (FBL) method of determining absorbance ΔA values as the reaction progresses.

Table 1. Variation of First-Order Rate constants k_{obs} (25 °C) with pH for the Reduction of *E. coli* Met-R2 (10−20μM) by Hydrazine, Total Concentration $[N_2H_4]_T$, Monitored at 370 nm, I = 0.100 M (NaCl)

pH	$[N_2H_4]_T$/mM	$10^4k_{obs}/s^{-1}$	pH	$[N_2H_4]_T$/mM	$10^4k_{obs}/s^{-1}$
6.64	56.3	1.04	8.33	49.1	10.1
6.99	50.8	1.71	8.47	20.9	5.11
7.50	48.9	4.13	8.47	40.5	10.2
7.85	52.3	6.39	8.47	50.5	12.5
8.10	51.6	8.93			

Table 2. Variation of First-Order Rate Constants k_{1obs}, k_{2obs}, and k'_{2obs} Obtained by the FBL Method (25 °C) for the Biphasic Reduction of Active *E. coli* R2 (10−20 μM) by Hydrazine, Total Concentration $[N_2H_4]_T$, Monitored at 370 nm, I = 0.100 M (NaCl)

pH	$[N_2H_4]_T$/mM	$10^4k_{1obs}/s^{-1}$	$10^4k_{2obs}/s^{-1}$	$10^4k'_{2obs}/s^{-1}$
6.60	65.3	1.28	0.47	0.55
7.10	49.1	2.70	0.75	0.82
7.71	65.6	3.4	0.95	1.19
7.68	64.6	7.3	1.66	2.53
8.0	49.3	7.5	1.51	2.73
8.21	60.9	10.4	2.44	4.05
8.41	34.5	8.7	1.74	2.84
8.46	22.0	5.8	1.19	2.14
8.50	14.8	4.4	1.14	1.48
	26.7	7.4	1.81	2.57
	40.8	10.2	2.32	3.45
	49.0	13.0	3.08	4.71
	49.3	13.6	2.80	4.40

Hydrazine Reduction of Active R2. A biphasic reaction is observed (scan spectra, Figure 2). First-order rate constants k_{1obs} and k_{2obs} obtained from absorbance changes at 370 nm are shown in Table 2. Rate constants at 410 nm are in good agreement (average ± spread 4−5%), and a linear dependence on $[N_2H_4]_T$ is observed (Figure 3), which yields second-order rate constants k_1 and k_2, respectively. The variation of k_1 with pH is shown in Figure 4, where points overlay with k_{Fe} obtained in the

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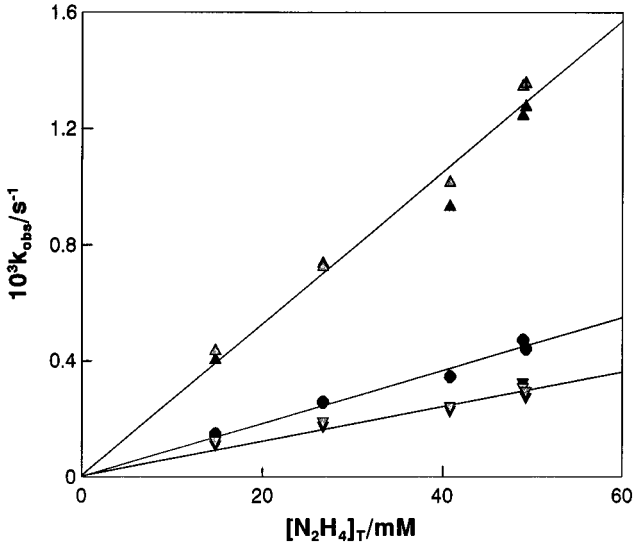

Figure 3. Dependence of first-order rate constants k_{obs} (25 °C) for the reaction of the *E. coli* R2 form of ribonucleotide reductase on total hydrazine, $[N_2H_4]_T$, at pH 8.50 (50 mM Tris/HCl), $I = 0.100$ M (NaCl). The rate constants (k_{1obs} and k_{2obs}, respectively) are shown for the first (△) and second stages (▽) of reaction monitored at 370 nm (open points) and 410 nm (solid points). The points ● are for k_{2obs} from the 410 nm decay using the fitted base-line (FBL) method.

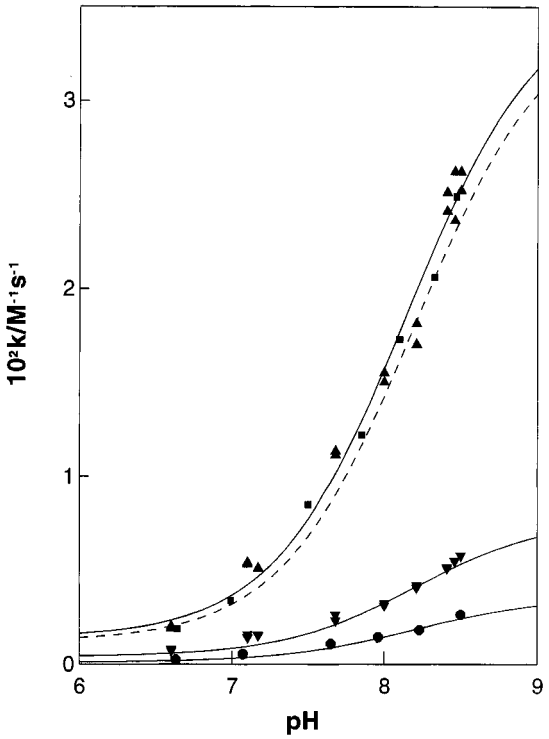

Figure 4. Comparison of rate constants (25 °C) with pH for the reaction of the active *E. coli* R2 ribonucleotide reductase, first phase (k_1; ▲) and second phase (k_2; ▼), alongside data for the Tyr122Phe variant (k_{Fe}; ●) and met-R2 (k_{Fe}; ■ with broken line), $I = 0.100$ M (NaCl).

previous section for met-R2 (Table 1). The first stage is therefore assigned to the single-stage reduction of met-R2 present (∼40%) as a component of active R2 preparations. The second phase of reaction involves concomitant decay of Tyr• and Fe^{III}_2 absorbance (Figure 2) and is here assigned to the 2-equiv reduction of Tyr• and one of the Fe^{III}'s of the Fe^{III}_2 center. Rate constants obtained from the 410 nm decay using the fitted base-line method emphasize the Tyr• decay and are defined as k'_{2obs}. Again a linear dependence on $[N_2H_4]_T$ is

Table 3. Summary of pK_a, k_O, and k_H values (25 °C) from the pH Dependence of Second-Order Rate Constants for the Reduction of Different *E. coli* R2 Forms with Hydrazine, $I = 0.100$ M (NaCl)

protein	rate constant	pK_a	$10^2k_O/M^{-1}\,s^{-1}$	$10^2k_H/M^{-1}\,s^{-1}$
met-R2	k_{Fe}	8.2(1)	3.5(3)	0.12(8)
active R2	k_1	8.15(9)	3.6(3)	0.14(6)
	k_2	8.2(1)	0.78(6)	0.04(1)
	k'_2	8.2(1)	1.3(1)	0.04(3)
Tyr122Phe R2	k_{Fe}	8.2(2)	0.36(6)	0.01(1)

Table 4. Variation of First-Order Rate Constants (25 °C) with pH for the Hydrazine Reduction of the *E. coli* Tyr122Phe R2 Variant of Ribonucleotide Reductase (10−20 μM), Monitored at 370 nm, $I =$ 0.100 M (NaCl)

pH	$[N_2H_4]_T$/mM	$10^4k_{obs}/s^{-1}$	pH	$[N_2H_4]_T$/mM	$10^4k_{obs}/s^{-1}$
6.63	61.2	0.15	8.50	19.6	0.55
7.07	77.6	0.42	8.50	29.0	0.80
7.65	61.3	0.67	8.50	45.2	1.13
7.96	57.1	0.82	8.50	63.1	1.73
8.23	63.9	1.17			

observed, but second-order rate constants k'_2 are some 50% greater than k_2 values.

Because of protonation, two forms of hydrazine are relevant, (7), and two redox steps (8) and (9) can be defined. The

$$N_2H_5^+ \overset{K_a}{\rightleftharpoons} N_2H_4 + H^+ \tag{7}$$

$$N_2H_4 + R2 \xrightarrow{k_O} \text{products} \tag{8}$$

$$N_2H_5^+ + R2 \xrightarrow{k_H} \text{products} \tag{9}$$

expression (10) can be derived for experimentally determined

$$k = \frac{k_O K_a + k_H[H^+]}{K_a + [H^+]} \tag{10}$$

rate constants k. A listing of parameters is given in Table 3. The errors alongside k_H indicate that this step makes little or no contribution to the reaction.

No absorbance changes were observed at ∼600 nm (see e.g. UV−vis spectra of $Fe^{II}Fe^{III}$ hemerythrin[20]), or elsewhere in the UV−vis range attributable to $Fe^{II}Fe^{III}$ formation and decay (Figure 2). From EPR studies, the formation of $Fe^{II}Fe^{III}$ is at low levels (∼5%).[17]

Hydrazine Reduction of the Tyr122Phe Variant. First-order rate constants k_{obs} are listed in Table 4. The pH variations are of a similar form (Figure 4) and reveal that rate constants are substantially (∼10 times) less than those obtained for the reduction of met-R2.

Phenylhydrazine Reduction of Active R2. Uniphasic reduction of the Tyr• only is observed (Figure 5), consistent with the reduction potential of 250 mV. The reductant absorbance changes were monitored at 410 nm. First-order rate constants k_{obs} are listed in Table 5 and give a linear dependence on $[C_6H_5N_2H_3]$. Rate constants using the FBL method were again larger by ∼40%. There is no dependence of k_{obs} on pH in the range 6.5−8.5. The pK_a for $C_6H_5N_2H_4^+$ of 5.27[28] indicates that $C_6H_5N_2H_3$ is the major form present. At pH 7.5, second-order rate constants of 0.184 ± 0.009 $M^{-1}\,s^{-1}$ are $>10^2$ times greater than those for the hydrazine reduction of Tyr• (and one Fe^{III}) of active R2.

Discussion

Active *E. coli* R2 samples have a met-R2 component, which has an Fe^{III}_2 center but no tyrosyl radical, generally (as in this

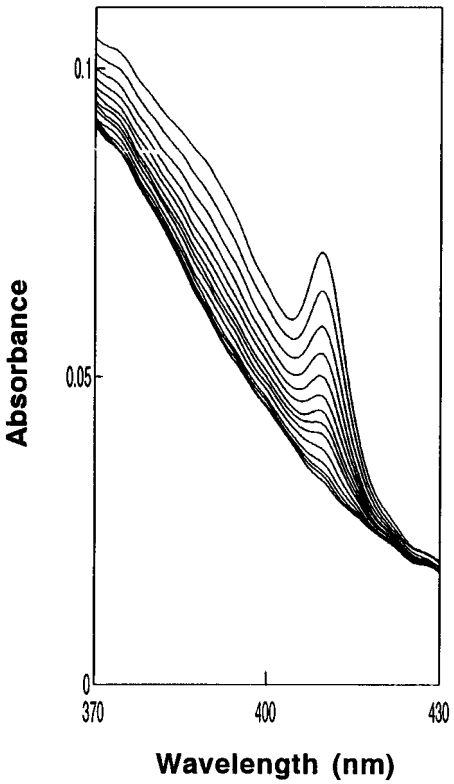

Figure 5. UV−vis scan spectra for the reaction (25 °C) of phenylhydrazine (9.9 mM) with the active *E. coli* R2 of ribonucleotide reductase at pH 7.6 (50 mM Tris/HCl), $I = 0.100$ M (NaCl). The first nine spectra were recorded 100 s and the next five at 300 s intervals.

Table 5. Variation of First-Order Rate constants k_{obs} (25 °C) with pH for the Phenylhydrazine Reduction of Active *E. coli* R2 Ribonucleotide Reductase (10−20 μM) Monitored at 410 nm, $I =$ 0.100 M (NaCl)

pH	$[C_6H_5N_2H_3]$/mM	$10^3 k_{obs}$ /s^{-1}	k_T/M^{-1} s^{-1}
6.5	14.7	2.93	0.20
7.0	14.7	2.64	0.18
7.6	9.9	1.85	0.19
7.6	16.8	3.08	0.18
7.6	24.6	4.74	0.19
8.2	14.8	2.68	0.18
8.5	14.7	2.49	0.17

work) close to 40% of the total protein. No preparations so far reported have <25% of this component.[7,21] The hydrazine reaction is unusual in that the met-R2 component is reduced prior to active Tyr•-containing protein. The kinetics of the first phase have been reproduced by separate experiments using met-R2 samples, and correspond to the $Fe^{III}_2 \rightarrow Fe^{II}_2$ reduction. In the second phase the UV−vis changes (Figure 2) indicate reduction of the Tyr• and Fe^{III}_2. The detection by EPR of Fe^{II}-Fe^{III} [17] suggests that there is a 2-equiv reduction of the Tyr• and one of the Fe^{III}'s.[17] No third stage of reaction is observed, and since the final spectrum corresponds to fully reduced Fe^{II}_2 (Figure 1) and the build-up of $Fe^{II}Fe^{III}$ does not exceed 5%, reduction of the second Fe^{III} overlays to a large extent the second stage. Build-up of $Fe^{II}Fe^{III}$ is also observed in the reduction of mouse R2 by hydrazine (9%).[35] Since there is no stable met-R2 form of mouse R2, it can be concluded that fully active R2 is the source of $Fe^{II}Fe^{III}$.

A reduction potential of −115 mV vs NHE ($2e^-$ process) has been reported for the Fe^{III}_2 center.[32] In view of the difficulty in identifying/retaining $Fe^{II}Fe^{III}$, it is likely that $Fe^{III}_2 \rightarrow Fe^{II}$-$Fe^{III}$ has a reduction potential more negative than −115 mV and that for $Fe^{II}Fe^{III} \rightarrow Fe^{II}_2$ is more positive. Estimates of the reduction potential for Tyr• range from 940 mV in small peptides[33] to 410 mV (pH 7.5) for the modified (3′-*S*-cysteinyltyrosine) tyrosyl radical in galactose oxidase.[34] From ref 32 the reduction potential for Tyr• in R2 has been estimated to be 1.00 ± 0.10 V, and from the range of values indicated, Tyr• is a much stronger oxidant than Fe^{III}_2. In previous studies with 1-equiv oxidants, there is no evidence of Fe^{III}_2 reduction occurring prior to Tyr•,[15] but this is clearly a possibility in the present studies. The reduction of the Fe^{III}_2 of met-R2 before the Tyr• of active protein is difficult to explain unless it relates to N_2H_4 behaving as a 2-equiv reductant, which provides an easier pathway for reduction. For the two-electron reduction of active protein, different reaction sequences are possible. Because $Fe^{II}Fe^{III}$ has been identified, reduction of Tyr• and one Fe^{III} is favored. However, an alternative would be for hydrazine to reduce $Fe^{III}_2 \rightarrow Fe^{II}_2$ in a process similar to that observed with met-R2. We note that the rate constants determined for the two processes differ by only a factor of ∼4. A rapid subsequent Tyr• oxidation of Fe_a could occur, yielding $Fe^{II}Fe^{III}$, which is then further reduced to Fe^{II}_2. The mechanism which we propose for the reaction of active *E. coli* R2 with hydrazine is therefore first phase, the reaction of met-R2, (11), and second

$$Fe^{III}_2 + N_2H_4 \rightarrow Fe^{II}_2 + N_2H_2 + 2H^+ \qquad (11)$$

phase, the overall reaction of active protein, (12). In both cases

$$Tyr^{\bullet}, Fe^{III}_2 + N_2H_4 \rightarrow TyrH, Fe^{II}Fe^{III} + N_2H_2 + H^+ \qquad (12)$$

the N_2H_2 product is capable of bringing about further reduction or undergoing spontaneous decay.

In this context, it is necessary to consider studies reporting the reduction of active *E. coli* R2 by 1,2-diazine,[36] which suggest that N_2H_2 is a stable moiety. In the latter, 1,2-diazine was generated *in situ* by hydrolysis of a 4 mM potassium azodicarboxylate at pH 8.5 (0.10 M Tris/HCl) under anaerobic conditions and in the presence of 1.15×10^{-5} M R2. The UV−vis absorbance changes over ∼2 h were assigned to the N_2H_2 reduction of R2. More recently the acid-catalyzed hydrolysis of azodiformate $(NCO_2)_2^{2-}$ to N_2H_2 was studied by stopped-flow spectrophotometry,[37] and the rate law is as indicated in (13). The mechanism proposed, (14) and (15) involving an

$$-d[(NCO_2)_2^{2-}]/dt = k[H^+][(NCO_2)_2^{2-}] \qquad (13)$$

intermediate X, gives $k = 1.2 \times 10^8$ M^{-1} s^{-1} at 25 °C, $I =$

$$(NCO_2)_2^{2-} + H^+ \rightarrow X \qquad (14)$$

$$X + H^+ \xrightarrow{\text{fast}} N_2H_2 + 2CO_2 \qquad (15)$$

0.11 M. Therefore, at pH 8.5, the half-life for N_2H_2 production is close to 2 s. The decay process, identified as a dihydrogen transfer (16),[38,39] yields hydrazine with a rate constant of $2.2 \times$

$$2N_2H_2 \rightarrow N_2H_4 + H_2 \qquad (16)$$

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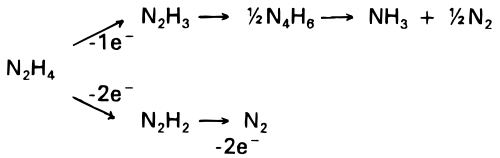
10^4 M^{-1} s^{-1},[40] with no dependence on pH between 1.5 and 5.1. Moreover, at pH 8, $(NCO_2)_2^{2-}$ decomposes in a few milliseconds to N_2H_2, which in turn decays in ~1 min.[41] All such observations are consistent with the instability of N_2H_2 above −180 °C[24,26] and suggest that N_2H_4 and not N_2H_2 is the reactive component in the studies with R2. Moreover, the EPR signal at resonances below $g = 2.00$ is very similar to that reported for the reaction of N_2H_4 with active R2.[17] The close agreement of EPR spectra with those observed for other $Fe^{II}Fe^{III}$ proteins,[42−49] as well as model $Fe^{II}Fe^{III}$ complexes,[50] leaves little doubt that $Fe^{II}Fe^{III}$ is formed.

Phenylhydrazine, although it reacts by a 2-equiv overall process (4), only reduces the $Tyr^•$ of active R2. The difference in behavior as compared to that of hydrazine is explained by the reduction potential of 250 mV vs NHE, which is too large for reduction of the Fe^{III}_2 center of R2 ($E^{o\prime} = -115$ mV) to occur.[32] The aromatic ring of phenylhydrazine presumably helps stabilize the 1-equiv oxidized product. Reactions in which the $C_6H_5^•$ radical participates and adds on to the product are known.[51]

A pathway for electron transfer has been proposed from the upper surface of the heart-shaped R2 close to the region of association with R1. Thus the conserved Trp-48 residue has been proposed as the lead-in group for electron transfer via Asp-237, His-118, Fe_a, and Asp-84 through to Tyr-122.[10,52] The proposed route is illustrated in ref 1. The aromatic group of the phenylhydrazine reductant may interact directly with Trp-48. On the other hand, hydrazine as a small-molecule reactant may need to access more closely the active site of R2 for a 2-equiv change to occur. From studies on inorganic electron transfer between coordination complexes, it has been concluded that 2-equiv changes take place by inner-sphere processes involving either atom or electron transfer.[53,54] No similar guidelines have yet emerged in the context of electron-transfer reactions of metalloproteins. Two small-molecule reactants O_2 and N_3^- are known to access and bind directly to the Fe^{II}_2 protein,[55,56] and it is reasonable therefore to consider hydrazine penetration in the present case. We have no direct evidence or information as to whether N_2H_2 generated reduces the second Fe^{III} or is released and another N_2H_4 enters or precisely what happens next. Interestingly, the bulkier 1-equiv phenylhydrazine reduction, which we assume reacts from the surface, is $>10^2$ times faster than that of hydrazine. The mechanistic behavior of hydrazine can be summarized by (17),[57] where N_2H_3, N_4H_6,



(17)

and N_2H_2 are transient intermediates. This scheme simplifies the situation, since with metal ion oxidants there is a dependence on whether the reaction is of the inner- or outer-sphere type. If N_2H_2 reacts further yielding N_2, then as in the regeneration of the $Tyr^•/Fe^{III}_2$ of R2 by O_2,[8,13,14,58] there is a one-electron imbalance to address. In the present studies, only ~15% regeneration of the original active R2 is observed on reacting with O_2 (Figure 2), consistent with N_2H_2 or N_2 remaining within the protein and blocking the O_2 oxidation. Alternatively, excess N_2H_4 may re-reduce regenerated protein.

The evidence provided by this study that $N_2H_5^+$ exhibits little or no reactivity with R2 (Figure 4) is in keeping with previous observations that charged reductants have difficulty in reducing the protein.[16,59] The finding that the Fe^{III}_2 is ~10 times less reactive in the Tyr122Phe R2 variant than in met-R2 is reflected in the midpoint potentials of −178 and −115 mV, respectively, reported for the reduction of Fe^{III}_2.[32] The difference in behavior indicates structural differences, possibly in the extent of hydrogen bonding close to the active site in the two forms.

In conclusion, a more quantitative and rigorous kinetic approach has been adopted in addressing important aspects of the hydrazine and phenylhydrazine reductions of the active *E. coli* R2 protein of ribonucleotide reductase. Studies on met-R2 leave no doubt as to the assignment of the first stage as the reaction of the met-R2 component of active R2 with hydrazine. The observation that active protein containing the strongly oxidizing $Tyr^•$ is reduced at a slower rate than the Fe^{III}_2 of met-R2 highlights a different reactivity pattern with 2-equiv reagents. The presence of Fe_a as a component in the pathway proposed for electron transfer from Trp-48 to $Tyr^•$ is noted, where 2-equiv reduction of Fe^{III}_2 may well occur prior to reduction of $Tyr^•$. Some degree of penetration of N_2H_4 to access the active site of R2 is also possible. It is of interest that strongly oxidizing $Tyr^•$ should actually inhibit the hydrazine reduction of active R2 as compared to met-R2 protein. The weaker reducing agent phenylhydrazine is unable to reduce the Fe^{III}_2, and 1-equiv reduction of the $Tyr^•$ is a $\sim 10^2$ faster process.

Acknowledgment. We wish to thank the University of The Orange Free State, Bloemfontein, for study leave (J.C.S.), the U.K. Science and Engineering Research Council (now EPSRC) for a research grant, and the North of England Cancer Research Campaign and British Council for generous support (to J.-Y.H.). We wish to acknowledge most helpful discussions with Dr. David Stanbury.

IC9515012

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