Reduction and Loss of the Iron Center in the Reaction of the Small Subunit of Mouse Ribonucleotide Reductase with Hydroxyurea†

S. Nyholm, L. Thelander, and A. Gräslund*

Department of Medical Biochemistry & Biophysics, University of Umeå, S-901 87 Umeå, Sweden

Received May 12, 1993; Revised Manuscript Received July 7, 1993®

ABSTRACT: Ribonucleotide reductase is a key enzyme for DNA synthesis in living cells, and the mechanisms for its reactions with inhibitors are of interest because the inhibitors are potential antiproliferative agents. Protein R2, the small subunit of mouse ribonucleotide reductase, contains a pair of μ-oxo-bridged ferric ions and a tyrosyl free radical in each of its two polypeptide chains. Light absorption spectroscopy was used to probe the reactions of these redox centers with hydroxyurea (HU), a potent inhibitor of iron containing ribonucleotide reductases. In *Escherichia coli* protein R2, HU reacts with the tyrosyl radical without affecting the iron center. In contrast to the case for the *E. coli* protein, HU destroys the specific absorbance bands of both the iron center and the radical on a similar time scale in mouse protein R2, and this is accompanied by release of iron from the protein. Anaerobic experiments with the iron chelator bathophenanthroline present during the HU reaction indicate that the iron is released from the mouse R2 protein in the ferrous form after treatment with HU. The reduced iron center, formed by reaction of Fe^{2+} with mouse apoprotein R2 under anaerobic conditions, was found to be much less stable than the native Fe^{3+} site in the presence of suitable iron chelators. The observations are of importance for understanding the mode of action of HU on mammalian cells and for the general question of the stability of the iron center of mouse protein R2 in different redox states.

Ribonucleotide reductase catalyzes the reduction of ribonucleotides to the corresponding deoxyribonucleotides, a reaction which is necessary for DNA replication. Mammalian cells, DNA viruses of the herpes virus group, and some prokaryotes (e.g., *Escherichia coli*) have a heterodimeric iron-containing enzyme of the $\alpha_2\beta_2$ type. Iron-containing ribonucleotide reductases are composed of two nonidentical subunits, proteins R1 and R2, each composed of two identical polypeptide chains. The large subunit, protein R1, binds substrates and allosteric effectors and contains the sulfhydryl groups directly responsible for the reduction of substrate during the enzymatic reaction. The small subunit, protein R2, contains in each of its two polypeptide chains a pair of antiferromagnetically coupled ferric ions and a tyrosyl free radical essential for enzymatic activity. A 1:1 complex of both subunits is necessary to form an active enzyme. [For reviews on the enzyme, see Thelander and Reichard (1979), Gräslund et al. (1985), Stubbe (1989), Eriksson and Sjöberg (1989), Thelander and Gräslund (1993)]. The crystal structure of protein R2 from *E. coli* has recently been elucidated (Nordlund et al., 1990).

The small subunit, protein R2, from mouse has been produced in large amounts in an *E. coli* recombinant system (Mann et al., 1991). The protein appears from the purification as an apoprotein essentially without iron and tyrosyl radicals. To regenerate the iron–tyrosyl radical center in the protein, Fe(II) was added to apoprotein R2 under anaerobic conditions. Then the solution was exposed to air, whereupon the diferric iron site is formed together with the tyrosyl radical (Ochiai et al., 1990).

The present study was undertaken to further probe the spectroscopic properties of the iron center of the mouse R2 protein and to investigate the reaction of the protein with hydroxyurea (HU). Hydroxyurea is a well-known inhibitor of the iron-containing ribonucleotide reductases. It specifically destroys the tyrosyl free radical, presumably through a one-electron reduction (Gräslund et al., 1985; Kjoller-Larsen et al., 1982; Karlsson et al., 1992; Lassmann et al., 1992). In the *E. coli* enzyme the HU reaction is known to involve only the tyrosyl radical, and the iron center is left intact, yielding the so-called *met* state of the protein (Sahlin et al., 1989). To obtain a direct action on the iron site of the *E. coli* protein, a more potent reductant like either diimide (Gerez et al., 1991) or dithionite together with a suitable mediator like benzyl viologen (Sahlin et al., 1989) is needed. For mammalian cells, however, it has been found that HU treatment leads to loss of iron, suggesting that HU is able to directly affect the iron center of mammalian ribonucleotide reductase (McClarty et al., 1990).

The present studies concern the reaction of HU with the iron center and tyrosyl radical in mouse protein R2, studied by light absorption spectroscopy. HU was found to react with the iron center as well as the free radical, resulting in a Fe^{2+} iron center. This observation led to further studies to probe the relative lability of the reduced iron center of mouse protein R2.

MATERIALS AND METHODS

Recombinant mouse R2 protein was prepared as previously reported (Mann et al., 1991). Regeneration of the iron–tyrosyl radical site was performed as previously described (Mann et al., 1991; Ochiai et al., 1990). The *E. coli* R2 protein was prepared as previously described (Sjöberg et al., 1986; Larsson & Sjöberg, 1986).

The proteins were kept and investigated in buffer solutions of 50 mM Tris and 100 mM KCl, pH 7.6. Protein

†This study was supported by grants from the Swedish Natural Science Research Council, the Magn. Bergvalls Stiftelse, the Lion's Cancer Research Foundation, the University of Umeå, and Bioteknikfonden at the University of Umeå.

* To whom correspondence should be addressed. Present address: Department of Biophysics, Stockholm University, S-10691 Stockholm, Sweden.

® Abstract published in *Advance ACS Abstracts*, October 1, 1993.

0006-2960/93/0432-11569$04.00/0

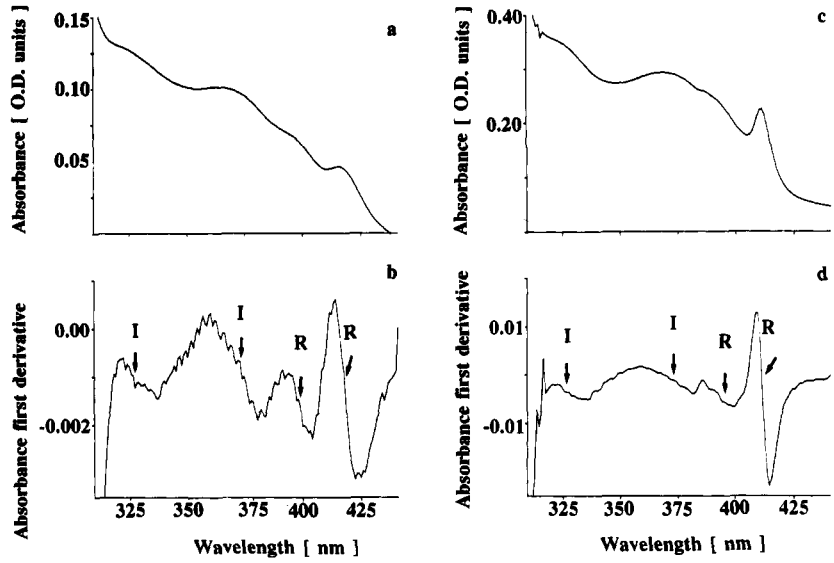

FIGURE 1: Optical absorption (a, c) and first-derivative spectra (b, d) of protein R2 from mouse (a, b) and *E. coli* (c, d) at 5 °C. For mouse protein the iron bands are indicated by "I" at 327 and 373 nm, and the radical bands are indicated by "R" at 398 and 417 nm. For *E. coli* protein the iron bands are indicated by "I" at 327 and 372 nm, and the radical bands are indicated by "R" at 395 and 411 nm.

concentrations were based on an absorbance index, $\epsilon_{280-310}$, of 62 000 M^{-1} cm^{-1} for the mouse R2 protein per polypeptide chain (Mann et al., 1991). In this study concentrations of protein R2 are given as polypeptide concentrations. Hydroxyurea was obtained from Sigma and used without further purification. Desferrioxamine was bought as Desferal (Ciba-Geigy AG, Basel, Switzerland) and used without further purification. Bathophenanthrolinedisulfonic acid was obtained from Sigma and used without further purification.

Light absorption spectra were recorded using a CARY 4 Varian spectrophotometer. The spectra were baseline corrected. First-derivative and difference spectra were calculated using the spectrophotometer software. The locations of midpoints of absorption bands were found by noting the maximum and minimum amplitudes of each derivative line and determining the wavelength that corresponded to the average of the maximum and minimum amplitudes. The temperature was controlled by using a thermostated water bath. In the anaerobic experiments the solutions were made anaerobic by exposure to argon gas for at least 1 h while the sample was kept at 0 °C. An anaerobic cuvette was used during the light absorption measurements. Except where stated differently, optical spectra during kinetic measurements were recorded in 1-min scans. Experiments were usually repeated at least twice, using protein from different preparations.

RESULTS

Optical Absorption Spectra. Optical spectroscopy (first derivative) was used to characterize the light absorption bands of the iron site and the tyrosyl radical for mouse protein R2 and to compare it to those of the *E. coli* enzyme. The use of the first derivative suppresses the effects of the protein light scattering, which is always present to some extent, and allows a more precise determination than previously (Mann et al., 1991) of the light absorption bands. Figure 1 shows the optical absorption and first-derivative spectra of R2 proteins from mouse and *E. coli* between 310 and 440 nm. The midpoints of the absorption bands assigned to the iron center and to the tyrosyl radical are indicated in the figure. The near coincidence particularly of the two iron bands in both proteins indicates a very close similarity between the geometries of the dimeric iron sites.

In other experiments we carefully quantitated the tyrosyl radical content of the mouse R2 protein and measured the light absorption of the sample. We found that the characteristic peak of the tyrosyl radical at 417 nm has $\epsilon = 5000$ M^{-1} cm^{-1}. This absorbance index includes a contribution also from the iron absorption bands.

Reaction of Protein R2 with Hydroxyurea. The reactivity of the iron–radical sites toward HU was investigated in experiments where HU was added to protein R2 from mouse and also for comparison to protein R2 from *E. coli*. The light absorption was monitored for different concentrations of HU and at different temperatures. Figure 2 shows spectra representing the difference before and after prolonged exposure to HU for the R2 proteins from mouse and *E. coli* at 15 °C. A 10 times lower concentration of HU was used for the mouse protein to compensate for its higher reactivity to HU and to give a similar time scale for the two proteins at this temperature. For comparison the figure also includes the optical spectrum of a model phenoxy radical produced chemically (Land et al., 1961). Whereas the difference spectrum of protein R2 from *E. coli* is essentially the same as that from the model radical, the mouse difference spectrum contains the iron bands as well.

Figure 3 shows a series of spectra obtained for mouse and *E. coli* protein R2 during treatment with HU. A lower temperature was used for the mouse protein to keep the time scales approximately the same. The results show that for the mouse protein R2 the radical and iron center light absorption bands are apparently affected on a similar time scale, in contrast to the *E. coli* protein where HU affects only the tyrosyl radical but leaves the iron center intact.

Labilization of Iron by the HU Reaction. Since HU may function as a reductant, a possible explanation for the induced loss of the ferric iron center light absorption could involve reduction of the iron ions. Furthermore, reduction of the iron center might make the iron ions less strongly bound to the protein, and they might be spontaneously lost or chelated out of the protein by HU. This hypothesis was tested by experiments using a combination of HU and iron chelators, desferrioxamine for Fe^{3+} and bathophenanthroline for Fe^{2+}. Separate studies using ^{59}Fe have shown (Nyholm et al., 1993) that the native iron site in protein R2 from mouse in the absence or presence of desferrioxamine gradually loses iron in a

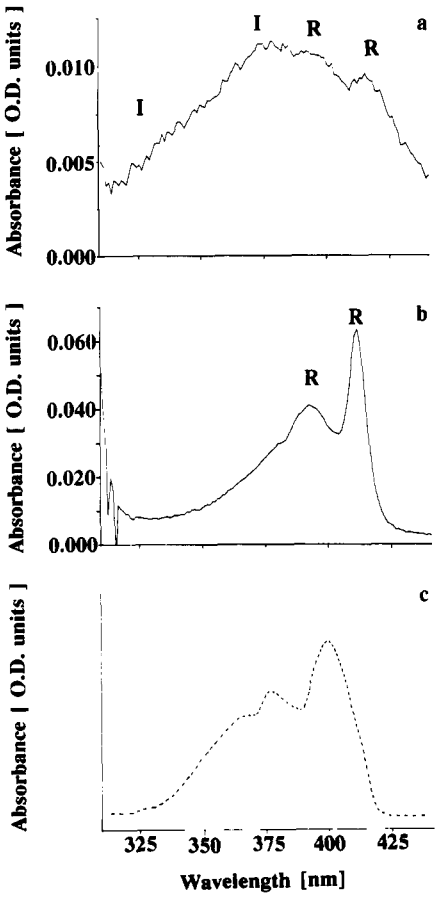

FIGURE 2: Difference light absorption spectra before and after hydroxyurea treatment of (a) 20 μM mouse protein R2 with 0.4 mM HU at 15 °C for 30 min and (b) 20 μM *E. coli* protein R2 with 4 mM HU at 15 °C for 30 min. (c) Spectrum of a chemically produced model 2,4,6-tri-*tert*-butylphenoxy radical in hexane [replotted from Land et al. (1961)].

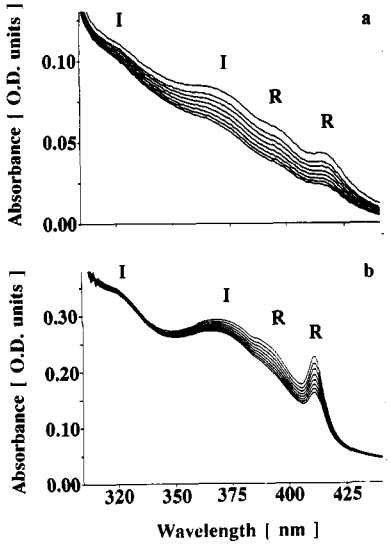

FIGURE 3: Light absorption spectra recorded every 200 s during the reaction of protein R2 with HU: (a) 20 μM mouse protein R2 reacted with 4 mM HU at 5 °C; (b) 20 μM *E. coli* protein R2 reacted with 4 mM HU at 15 °C. Each spectrum was recorded over 180 s.

temperature-dependent process. At 20 °C about 25% of the total iron is lost in 30 min from the native protein. The total amount lost may be affected by the presence of small amounts of unspecifically bound iron. The experiments to be described below were performed at temperatures of 15 °C or lower.

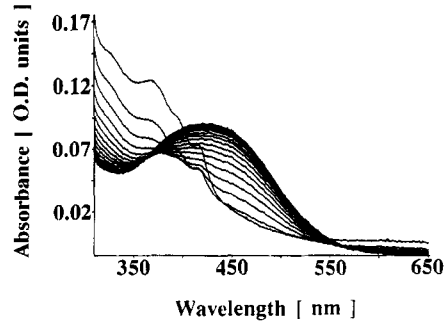

FIGURE 4: Light absorption spectra recorded every 30 min during the reaction of 18 μM mouse protein R2 with 4 mM HU and 100 μM desferrioxamine at 5 °C.

Figure 4 shows that treatment of mouse protein R2 (18 μM in polypeptide chain concentration) with a combination of HU and desferrioxamine leads to changes in the light absorption spectrum where the iron and radical bands are lost, in parallel with the growth of an absorption band at 430 nm, typical of the ferrioxamine complex. Quantitation of the ferrioxamine complex light absorption (ϵ_{430} = 2500 M^{-1} cm^{-1}; Goodwin & Whitten, 1965) gives a final iron concentration of 40 μM. This shows that iron leaves the protein in the presence of HU and desferrioxamine. The quantity is in good agreement with the amount of iron contained in the native iron site, assuming 2 iron ions per polypeptide chain (2 × 18 μM). A parallel experiment with desferrioxamine at 15 °C in the absence of HU showed a moderate loss of iron (about 13% of the total added iron) into the solution (Figure 6b). This iron loss is similar in magnitude to the spontaneous loss from native mouse R2 without any added desferrioxamine under similar conditions (Nyholm et al., 1993).

Desferrioxamine is a potent chelator of ferric iron. One way to produce a ferrioxamine complex quantitatively at neutral pH is to mix desferrioxamine prepared in air with a solution of Mohr's salt ($H_3NFe^{2+}SO_4^{2-}$) prepared anaerobically. Despite the fact that Mohr's salt contains ferrous iron, the ferric complex with desferrioxamine is formed instantaneously and quantitatively [cf. Goodwin and Whitten (1965)]. Therefore, the experiment with HU and desferrioxamine does not distinguish between iron leaving the protein as Fe^{2+} or Fe^{3+}.

To probe further into this question, we performed experiments using bathophenanthroline, a potent Fe^{2+} chelator with a typical light absorption at 535 nm (ϵ = 22 000 M^{-1} cm^{-1}; Blair & Diehl, 1961; Atkin et al., 1973). Figure 5a shows light absorption spectra observed when mouse protein R2 (184 μM) was incubated under anaerobic conditions with bathophenanthroline in the presence of HU (4 mM). Figure 5b shows a time curve for the increase in absorbance at 535 nm as a function of time. In the absence of HU, bathophenanthroline has a negligible effect on protein R2 (Figure 5b). In the presence of HU, the typical Fe^{2+}–bathophenanthroline light absorption appears in parallel with the loss of iron–free radical light absorption (Figure 5a). Since no other reductant was present in the system, the direct explanation of these results is that iron leaves mouse protein R2 as Fe^{2+} after treatment with HU. In this case the final concentration of the iron complex was only about 60% of that expected (the expected concentration being twice the concentration of protein R2 polypeptide). One explanation for the rather low iron complex concentration may be that the sample still contained a small amount of oxygen. The Fe^{2+} leaving the protein may have become oxidized to Fe^{3+} before having time to react with bathophenanthroline to form the colored complex.

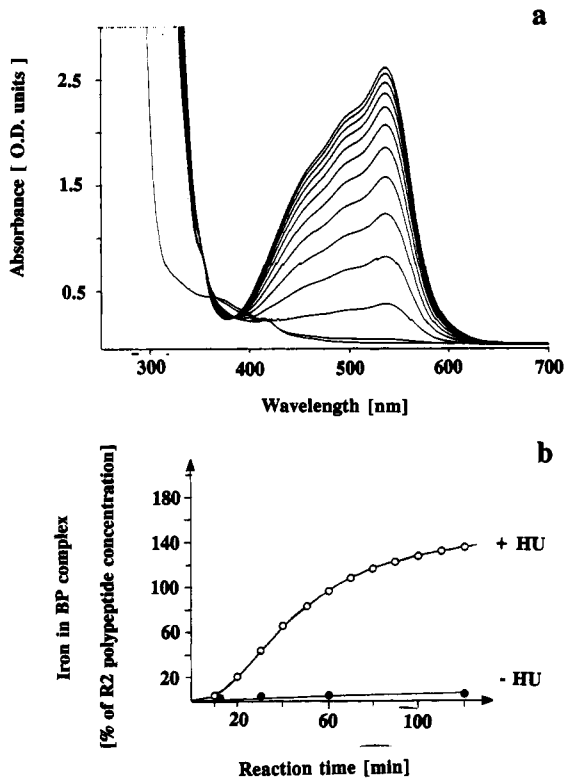

FIGURE 5: (a) Light absorption spectra at 15 °C of 87 μM mouse protein R2, 1 mM bathophenanthroline, and 4 mM HU, recorded every 10 min under anaerobic conditions. (b) Quantitative evaluation of Fe^{2+}–bathophenanthroline complex formation in the presence of 4 mM HU (○) (data from Figure 5a) and in the absence of HU (●). The results in the absence of HU were obtained with 122 μM protein R2 and 1 mM bathophenanthroline at 15 °C. The vertical scale is given as percent of R2 polypeptide concentration. The amount of iron complex was estimated from the absorbance at 535 nm.

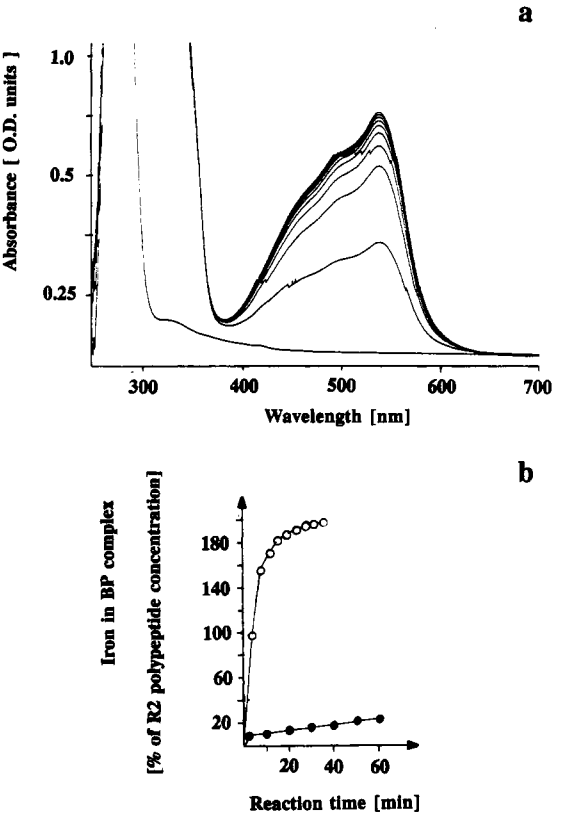

FIGURE 6: (a) Light absorption spectra at 15 °C of 23 μM mouse apoprotein R2 reacted under anaerobic conditions with 46 μM Fe^{2+} and incubated for 10 min at 25 °C before addition of 1 mM bathophenanthroline. The spectra were recorded at 4-min intervals under anaerobic conditions. (b) Quantitative evaluation of Fe^{2+}–bathophenanthroline complex formation from the reduced iron site in mouse protein R2 (○) (data of Figure 6a) and formation of Fe^{3+}–desferrioxamine complex from native protein R2 (82 μM) in air, in the presence of 1 mM desferrioxamine at 15 °C (●). The amount of iron in the desferrioxamine complex was estimated from the absorbance at 430 nm. The vertical scale is given as in Figure 5b.

Indeed, adding Fe^{2+} directly to an argon-flushed solution of bathophenanthroline treated similarly to the protein also results in only about 60% recovery of the added iron in the bathophenanthroline complex, probably reflecting the amount of remaining oxygen in the solution (data not shown).

Formation and Labilization of a Reduced Iron Center. Since the results with HU suggested that a reduced iron center in mouse protein R2 is destabilized relative to the native center, we performed further experiments to probe the stability of a reduced iron center formed in a more straightforward way in mouse protein R2. Argon-flushed apoprotein R2 was reacted with Fe^{2+} and incubated for 10 min, whereupon bathophenanthroline was added to the mixture. All manipulations were done anaerobically. Figure 6a shows a series of light absorption spectra taken in the course of the reaction. The bathophenanthroline shows a reaction with Fe^{2+} which accounts for all the added Fe^{2+}. Figure 6b shows the time course of the absorbance at 535 nm. The Fe^{2+}–bathophenanthroline complex is formed with a half time of about 4 min at 15 °C, indicating a relatively rapid loss of the Fe^{2+} center in mouse R2. At the same temperature the loss of iron from the native enzyme (in the absence of hydroxyurea) in the presence of the same concentration of desferrioxamine or bathophenanthroline was negligible (Figures 6b and 5b). In the absence of protein the reaction between bathophenanthroline and Fe^{2+} is instantaneous under the present conditions (data not shown).

Kinetics of the Mouse Tyrosyl Radical Reaction with HU. To investigate the kinetics of the reaction of mouse protein R2 with HU, the time dependence of the absorbance at 417 nm was studied. We evaluated an absorbance difference, $A(t)$, between the absorbance at 417 nm and a straight line drawn through the absorbance curve at 405 and 434 nm (approximately a tangent to the absorbance spectrum). We considered this absorbance difference to be proportional to the amount of tyrosyl radical present, since the total absorbance at 417 nm contains an unknown contribution from the iron center. Figure 7 shows an example of $A(t)$ as a function of time. The $A(t)$ curves were found to be close to exponential in shape, and pseudo-first-order rate constants could be evaluated. The second-order rate constant, k_2, was obtained by division by the HU concentration.

In a van't Hoff graph, the logarithm of the temperature dependence of the second-order rate constant, k_2, for the reaction of mouse R2 protein (10 μM) with 4 mM HU was found to be close to a linear function of the inverse absolute temperature in the temperature range 5–25 °C (data not shown). At 25 °C the rate constant for the hydroxyurea reaction with mouse R2 is 1.45 M^{-1} s^{-1}, about 3 times faster than the corresponding rate constant reported for the *E. coli* protein (Lam et al., 1990; Karlsson et al., 1992; Lassmann et al., 1992). The dependence of ln k_2 on the inverse absolute temperature was fitted to a straight line using linear regression. From the slope of the line we evaluated an activation energy of 66 kJ mol^{-1}.

In other experiments the reaction rate constant k_2 was studied as a function of HU concentration at a low temperature, 5 °C. For concentrations of HU between 4 and 20 mM we

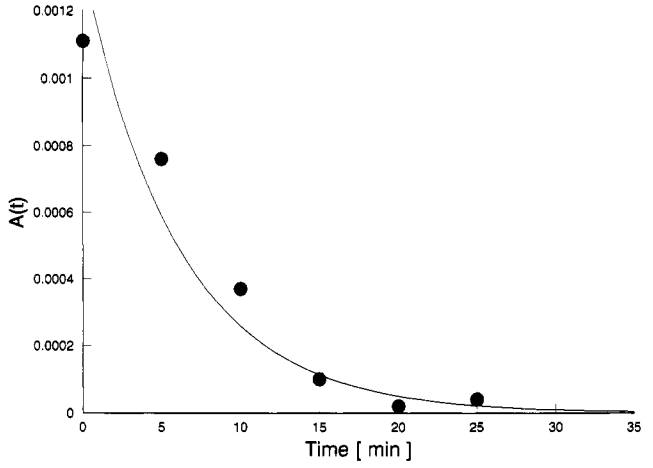

FIGURE 7: Time dependence of the tyrosyl radical absorbance in the reaction of 10 μM mouse R2 protein with 4 mM HU at 15 °C. The absorbance difference, $A(t)$, was evaluated as the difference between the absorbance at 417 nm and a straight line through the absorbance at 405 and 434 nm, approximately a tangent to the absorbance spectrum, as described in the text. The exponential curve $A(t) = 0.00134e^{-0.165t}$, where t was measured in minutes, was fitted to the experimental points by a regression procedure.

found no significant differences in the evaluated rate constants (data not shown), indicating that there are no obvious saturation effects for the reaction of HU in this concentration range.

Previous studies of the *E. coli* protein R2 have shown that the room temperature reaction with HU proceeds without apparent saturation up to at least 450 mM HU (Lassmann et al., 1992).

DISCUSSION

The most important new observations in this study are the following: (1) The iron site in mouse protein R2 reacts with and is most likely reduced by HU, showing that the iron center is more easily affected by external agents than is the case for the *E. coli* protein (Figures 2 and 3). This could simply reflect a more exposed site for the iron center in the mouse protein [cf. Kjöller-Larsen et al. (1982)], or it could be related to different redox properties of the iron–free radical sites in the two proteins. (2) The protein-bound iron in the reduced form of mouse protein R2 (formed either by reaction with HU or by direct anaerobic addition of Fe^{2+} to apoprotein) is much more labile than in the native diferric oxo-bridged form and equilibrates with the medium so that it can be chelated and detected in the presence of iron chelators (Figures 4–6).

The close similarities between the iron bands in the mouse and *E. coli* R2 proteins as revealed in the light absorption derivative spectra (Figure 1) show that the geometries of the iron sites should be quite similar. Previous electron spin resonance (ESR) studies have shown a significant difference between the mouse and *E. coli* R2 proteins in the interaction between the iron sites and the tyrosyl free radicals, evidenced by the differences in the temperature-dependent ESR relaxation properties. The difference has been interpreted in terms of a different structural arrangement, possibly a smaller distance between the iron site and the free radical in the mouse protein, compared to the *E. coli* one (Mann et al., 1991). We can now propose that the observed ESR differences should be due to a different arrangement of the radical relative to the iron site, most likely a different distance between them, rather than to geometrical differences in the iron sites themselves. The extinction coefficient of 5000 M^{-1} cm^{-1} for the 417-nm band characteristic of the radical-containing enzyme agrees fairly well with the corresponding value reported for the *E. coli* protein (6600 M^{-1} cm^{-1}; Peterson et al., 1980). The somewhat lower value found for the mouse protein could be related to the fact that its absorption band appears less narrow.

All available data indicates that iron leaves mouse protein R2 as Fe^{2+} after the reaction with HU. These results indicate that iron in a reduced iron site is relatively weakly bound to the protein. The relative lability of the reduced iron site is further emphasized by the observation that anaerobic addition of Fe^{2+} leads to a state where the iron can be easily and rapidly chelated out and quantitatively detected in the presence of the ferrous iron chelator bathophenanthroline (Figure 6). In contrast, the ferric iron chelator desferrioxamine has a negligible effect on the native iron site under similar conditions (Nyholm et al., 1993; Figure 6b). The moderate loss of iron in the presence of desferrioxamine is comparable with that occurring spontaneously in a temperature-dependent process in mouse protein R2 (Nyholm et al., 1993). There is obviously a big difference in stability for Fe^{3+} and Fe^{2+} in the metal site.

The kinetic results on the reaction of HU with the tyrosyl radical of mouse protein R2 are in good agreement with previous observations showing that the mouse protein is more sensitive than the *E. coli* one to the inhibitory activity of HU (Kjoller- Larsen et al., 1982). Despite the higher rate constant for the reaction with the mouse protein, the activation energy obtained from its temperature dependence (66 kJ mol^{-1}) is quite similar to that determined for the *E. coli* protein (67 kJ mol^{-1}; Karlsson et al., 1992), suggesting that the reaction pathways may be similar in the two cases.

We searched for possible evidence of saturation at high HU concentrations in the reaction with protein R2. If saturation had been observed, it would have indicated the presence of a particular saturable binding site for HU at the protein surface. So far we have not found any evidence for this, and the present results suggest the simpler model in which HU does not require a particular binding site to reduce the protein. The reaction kinetics should be modulated by protein dynamics, mediating the access of the reductant or electron transport to the iron center–radical site. For the *E. coli* enzyme it has been shown that a complete enzyme system including a bound substrate or product makes the radical 10 times more susceptible to HU (Karlsson et al., 1992).

In conclusion, the present study demonstrates the ability of HU to reduce the iron center as well as the tyrosyl radical of mouse protein R2 and the subsequent lability of the reduced iron site and facilitated loss of iron from the protein. These results explain why mouse ribonucleotide reductase treated with HU is easily reactivated under assay conditions (with the reductant dithiothreitol present) without the special iron chelation procedure needed for the reactivation of the R2 protein from *E. coli* (Atkin et al., 1973). The observed lability of the reduced iron–radical site also agrees with the previous observations that iron and oxygen are continuously required for mammalian ribonucleotide reduction (Thelander et al., 1983) and that iron is lost upon HU treatment of mammalian cells (McClarty et al., 1990). These observations also raise further questions regarding the enzyme properties under turnover conditions, which might involve a transient reduction of the iron center.

From a medical point of view, the understanding of the reactions of ribonucleotide reductase with potent inhibitors of the enzyme is important, since ribonucleotide reductase is a potentially important target for new antitumor and antiviral drugs.

REFERENCES

Atkin, C. L., Thelander, L., Reichard, P., & Lange, G. (1973) *J. Biol. Chem. 248,* 7464–7472.

Blair, D., & Diehl, H. (1961) *Talanta 7,* 163–174.

Eriksson, S., & Sjöberg, B.-M. (1989) in *Allosteric Enzymes* (Hervé, G., Ed.) pp 189–215, CRC Press, Boca Raton, FL.

Gerez, C., Gaillard, J., Latour, J. M., & Fontecave, M. (1991) *Angew. Chem., Int. Ed. Engl. 30,* 1135–1136.

Goodwin, J. F., & Whitten, C. F. (1965) *Nature 205,* 281–283.

Gräslund, A., Ehrenberg, A., & Thelander, L. (1982) *J. Biol. Chem. 257,* 5711–5715.

Gräslund, A., Sahlin, M., & Sjöberg, B.-M. (1985) *Environ. Health Perspect. 64,* 139–149.

Karlsson, M., Sahlin, M., & Sjöberg, B.-M. (1992) *J. Biol. Chem.* 267, 12622–12626.

Kjoller-Larsen, I., Sjöberg, B.-M., & Thelander, L. (1982) *Eur. J. Biochem. 125,* 75–81.

Lam, K., Fortier, D., Thomson, J., & Sykes, G. (1990) *J. Chem. Soc., Chem. Commun. 1990,* 658–660.

Land, E. J., Porter, G., & Strachan, E. (1961) *Trans. Faraday Soc. 57,* 1885–1893.

Larsson, Å., & Sjöberg, B.-M. (1986) *EMBO J. 5,* 2037–2040.

Lassmann, G., Thelander, L., & Gräslund, A. (1992) *Biochem. Biophys. Res. Commun. 188,* 879–887.

Mann, G., Gräslund, A., Ochiai, E.-I., Ingemarson, R., & Thelander, L. (1991) *Biochemistry 30,* 1939–1947.

McClarty, G. A., Chan, A. K., Choy, B. K., & Wright, J. A. (1990) *J. Biol. Chem. 265,* 7539–7547.

Nordlund, P., Sjöberg, B.-M., & Eklund, H. (1990) *Nature 345,* 593–598.

Nyholm, S., Johansson, A., Mann, G. J., Bergeron, R. J, Gräslund, A., & Thelander, L. (1993) *J. Biol. Chem.* (in press).

Ochiai, E.-I., Mann, G., Gräslund, A., & Thelander, L. (1990) *J. Biol. Chem. 265,* 15758–15761.

Peterson, L., Gräslund, A., Ehrenberg, A., Sjöberg, B.-M., & Reichard, P. (1980) *J. Biol. Chem. 255,* 6706–6712.

Sahlin, M., Gräslund, A., Petersson, L., Ehrenberg, A., & Sjöberg, B.-M. (1989) *Biochemistry 28,* 2618–2625.

Sjöberg, B.-M., Hahne, S., Karlsson, M., Jörnvall, H., Göransson, M., & Uhlin, B.-E. (1986) *J. Biol. Chem. 261,* 5658–5662.

Söderman, K., & Reichard, P. (1986) *Anal. Biochem. 152,* 89–93.

Stubbe, J. (1989) *Adv. Enzymol. Relat. Areas Mol. Biol. 63,* 349–419.

Thelander, L., & Reichard, P. (1979) *Annu. Rev. Biochem. 48,* 133–158.

Thelander, L., & Gräslund, A. (1993) in *Metal Ions in Biological Systems* (Sigel, H., & Sigel, A., Eds.) Vol. 30, M. Dekker, Inc., New York (in press).

Thelander, M., Gräslund, A., & Thelander, L. (1983) *Biochem. Biophys. Res. Commun. 110,* 859–865.