Escherichia coli and herpes-simplex-virus ribonucleotide reductase R2 subunit

Compared reactivities of the redox centres

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Protein R2, the small subunit of ribonucleotide reductase, contains a diferric centre and a tyrosyl radical absolutely required for enzyme activity. The reduction of the tyrosyl radical and the mobilization of the iron centre result in the inhibition of the enzyme and thus of DNA synthesis. The chemical reactivity of the iron-radical centre of *Escherichia coli* and herpes simplex virus has been studied by u.v.–visible and e.p.r. spectroscopies. The tyrosyl radical is efficiently scavenged by hydroxamic acids and phenols during reactions controlled by steric hindrance

and hydrophobic interactions. The reaction with *o*-disubstituted phenols yields the corresponding diphenquinones. The reactivity of the bacterial radical greatly contrasts with that of the viral radical, and the iron centre in herpes-simplex-virus R2 is much more labile than that in *E. coli* R2, as shown from the facile mobilization of iron by chelators such as catechol. These results suggest that the active sites of the two enzymes are significantly different and might be useful for designing new antiviral agents.

INTRODUCTION

Ribonucleotide reductase is an essential enzyme in all living organisms, including bacteria, viruses and mammals. It provides the cell with the correct concentrations of deoxyribonucleotides required for DNA synthesis [1]. In *Escherichia coli*, in herpes simplex viruses (HSV) and in mammals, the enzyme consists of a 1:1 complex of two non-identical homodimer subunits, named R1 and R2 [2,3]. Protein R2 contains one binuclear non-haem iron centre and a stable tyrosyl radical within each polypeptide chain [2,3].

The iron centre is essential during the process of post-translational activation of protein R2, i.e. the generation of a free radical at a specific tyrosine position. On the other hand, the tyrosyl radical is absolutely necessary for ribonucleotide reduction. Hydroxyurea is an excellent scavenger of this protein radical and therefore a good inhibitor of ribonucleotide reductase and DNA synthesis [2]. It is actually used as an anticancer drug in clinics. Studies on the inhibition of *E. coli* reductase by other radical scavengers *in vitro*, such as hydroxamic acids, hydroxylamines and hydrazines, have shown that the rates of electron transfer to the tyrosyl radical were controlled by the steric hindrance and the hydrophobicity of the inhibitors [5,6].

The current information on the structure and the chemical reactivity of the redox centres of protein R2 essentially relies on studies on *E. coli* reductase. The three-dimensional structure of *E. coli* protein R2 crystals has been recently determined by X-ray diffraction and provides a great source of information concerning the chemical environment of the iron-radical site [4]. Whether this information can be extrapolated to proteins R2 from other species is not known. For example, much less is known about the accessibility and reactivity of the HSV tyrosyl radicals and iron centres. In fact, HSV R2 protein was, until very recently, not available in large quantities required for chemical and structural

studies [N. Lamarche, B. Massie, M. Atta, M. Fontecave, C. Guilbault and Y. Langelier, unpublished work; 7].

Here we present a systematic comparison of recombinant *E. coli* and HSV proteins R2 in terms of the reactivity of their tyrosyl radical and their iron centres towards electron donors, hydroxamic acids, phenols and catechols. This may be useful for improving our understanding of the topology of the viral active site.

MATERIALS AND METHODS

Materials

The hydroxamic acids were generously given by I. Kjöllér Larsen (Royal Danish School of Pharmacy, Copenhagen, Denmark). Diphenquinones [8] and vitamin E analogues [9] were prepared as previously described. Stock solutions of hydrophobic organic compounds were prepared in dimethyl sulphoxide (DMSO). Protein R2 from *E. coli* was purified from overproducing strains *E. coli* [10]. *E. coli* apoR2 was prepared by treatment of protein R2 with 8-hydroxyquinolinesulphonate [11]. *E. coli* metR2 was prepared from R2 by treatment with hydroxyurea [12]. HSV type 2 protein R2 was purified from cells infected with the recombinant adenovirus Ad5BM5 R2 (N. Lamarche, B. Massie, M. Atta, M. Fontecave, C. Guilbault and Y. Langelier, unpublished work). Obtained in a radical-free form, HSV R2 was activated by treatment with 50 mM dithiothreitol (DTT) under aerobic conditions and then dialysed [N. Lamarche, B. Massie, M. Atta, M. Fontecave, C. Guilbault and Y. Langelier, unpublished work; 13].

Spectroscopic measurements

E.p.r.

Protein R2 (1–2 mg/ml) was dissolved in 150 μ l of 0.1 M

Tris/HCl buffer, pH 7.5, in an Eppendorf tube at 4 °C. Just after the addition of a small volume of the radical scavenger solution (final concentration of DMSO < 1%), the contents of the Eppendorf tube were vortex-mixed and immediately transferred to an e.p.r. tube. Incubation was carried out in the e.p.r. tube at 37 °C and was stopped at time intervals by freezing the sample at liquid-N₂ temperature. Preparations of bacterial and viral R2 were stable during freezing and thawing. First-derivative e.p.r. spectra were recorded on a Varian E102 spectrometer. The loss of the radical was quantified from the amplitude of the $g = 2.00$ signal. The microwave power was set at 10 mW for *E. coli* R2 and 20 mW for HSV R2.

Light-absorption spectra

These were recorded on a UVIKON 930 spectrophotometer. The spectroscopic cuvette was first filled with 1 ml of 0.1 M Tris/HCl buffer containing the reductant. The optical spectrum of the solution was recorded and stored in the spectrophotometer memory for subtraction from all subsequent spectra. The reaction was started by addition of a small volume of protein R2 solution (1–2 mg/ml final concn.), and optical spectra were recorded at time intervals. Some experiments were carried out under anaerobic conditions in spectroscopic cuvettes capped with a rubber septum and deaerated by flushing for 2 h with argon. Addition of deaerated solutions of protein R2 and scavengers were made through the septum with gas-tight syringes that had been thoroughly washed with deoxygenated water.

RESULTS

Reduction of the tyrosyl radical by hydroxamic acids and phenols

The reduction of the tyrosyl radical of *E. coli* or HSV protein R2 by hydroxamic acids and phenols was monitored by e.p.r. spectroscopy (Tables 1 and 2). Indeed, the amount of radical can be easily determined from the amplitude of its characteristic e.p.r. signal at $g = 2.00$ at liquid-N₂ temperature. As shown in Table 1, the reactivity of substituted hydroxamic acids greatly decreased with increased steric hindrance. This was much less pronounced during reduction of the HSV R2 radical. On the other hand, the *E. coli* R2 radical was relatively more sensitive to phenyl-substituted hydroxamic acids than the HSV R2 radical (Table 1). As an example, the phenyl derivative was more active than the ethyl derivative with respect to the *E. coli* radical, but not to the HSV radical. Experiments with phenols (Table 2) again demonstrated a large difference between the two proteins. While phenols reacted with the radical of *E. coli* protein R2 in the order: 2,4,6-trimethylphenol > 2,6-dimethylphenol > phenol > 4-acetylaminophenol (paracetamol), the reverse order was obtained during reaction with the radical of HSV R2 (Table 2). Vitamin E analogues, previously described as excellent radical-scavenger phenols [9], were poor inhibitors of the bacterial and viral enzyme (results not shown). Catechol is much more active than phenol. Quercetin, a natural flavonoid catechol, was found to be a good inhibitor of HSV R2.

R2-dependent oxidation of substituted phenols to quinones

The reaction of *E. coli* protein R2 with radical scavengers can also be monitored spectrophotometrically, since the protein R2 u.v.–visible spectrum contains absorption bands characteristic of the diferric complex and the tyrosyl radical, at 360–370 nm and

410 nm respectively (Figure 1, spectrum 1). Figure 1 shows the u.v.–visible spectrum of the incubation mixture containing 1 mg/ml protein R2 and 10 mM 2,6-dimethylphenol in 0.1 M Tris buffer, pH 7.5, under aerobic conditions. We observed that the disappearance of the 410 nm peak, which proved the quick scavenging of the tyrosyl radical, was paralleled with the appearance of a new absorption band at 420 nm. The shape of this

Table 1 Reactivity of substituted hydroxamic acids (RCONHOH) towards the tyrosyl radical of protein R2

Assays were carried out as described in the Materials and methods section. The relative e.p.r. amplitude varies from 100 (no scavenging of the radical) to 0 (total scavenging of the radical). The concentration of radical scavengers was 1 mM and the incubation time was 20 min (*E. coli*) or 10 min (HSV).

R	Relative e.p.r. amplitude	
	<i>E. coli</i>	HSV
NH ₂	19	5
CH ₃	81	30
C ₂ H ₅	> 95	35
C ₄ H ₉	> 95	45
C ₆ H ₅	85	59
C ₆ H ₅ -NH-	71	90
C ₆ H ₅ -CH ₂ -	> 95	67

Table 2 Reactivity of phenols towards the radical of protein R2

The concentration of radical scavengers was 1 mM and the incubation time was 10 min.

Compound	Relative e.p.r. amplitude	
	<i>E. coli</i>	HSV
Phenol	86	35
2,6-Dimethylphenol	29	66
2,4,6-Trimethylphenol	7	75
4-Acetylaminophenol	> 95	13
2,6-Dichlorophenol	> 95	84
2,6-Dimethoxyphenol	90	78
Catechol	0	0
Quercetin	89	25

Figure 1 Reaction of *E. coli* protein R2 with 2,6-dimethylphenol

R2 (1 mg/ml) was incubated with 10 mM 2,6-dimethylphenol under aerobic conditions. The light absorption spectrum was recorded after 30 s (1), 10 min (2) and 20 min (3).

Figure 2 Reaction of *E. coli* protein R2 with catechol

Protein R2 (2 mg/ml) was incubated with 1 mM catechol at pH 7.5, under anaerobic conditions. The light-absorption spectrum of the reaction mixture was recorded after 30 s (1), 10 min (2), 30 min or 2 h (3). The inset shows the spectrum after 3 h incubation of *E. coli* protein R2 with 10 mM catechol at pH 8.8.

Figure 3 Reaction of HSV protein R2 with catechol

The reaction was carried out under conditions described in Figure 2. The light-absorption spectrum of the reaction mixture was recorded after 0 s (1), 30 s (2), 5 min or 2 h (3).

band and the absorption wavelength are identical with those of an authentic sample of 3,5,3',5'-tetramethyldiphenoxinone, a well-known oxidation product of 2,6-dimethylphenol [8]. The intensity of this band then slowly decreased owing to the limited solubility of the quinone in aqueous solutions. No 420 nm band appeared in parallel with the loss of the 410 nm peak when the reaction was carried out under anaerobic conditions. The formation of the diphenoxinone was absolutely dependent on the presence of the tyrosyl radical, since no 420 nm band could be detected during the reaction between 2,6-dimethylphenol and metR2, the inactive iron-containing and radical-free form of R2. Reaction of 2,4,6-trimethylphenol with protein R2 led also to a rapid reduction of the tyrosyl radical, but with no formation of any absorbing product. When 2,6-dimethoxyphenol, another *o*-disubstituted phenol, was used as a scavenger of protein R2 radical, again an absorption band, at 460 nm, characteristic of the 3,5,3',5'-tetramethoxydiphenoxinone, appeared during the disappearance of the 410 nm peak (results not shown).

Mobilization of iron from R2 by catechol

Some of the radical scavengers used in the present study are also iron chelators. Examples are hydroxamic acids and catechol. In order to see whether they may also gain access to the iron site within the polypeptide chain and chelate off the ferric ions, we studied the reaction of *E. coli* and HSV proteins R2 with catechol. This was carried out under anaerobic conditions to avoid catechol autoxidation. The mobilization of iron was monitored spectrophotometrically, since iron-catecholates have characteristic intense charge-transfer bands in the visible region. The position of these bands is known to depend on the pH of the solution: 490 nm at pH 8.8 and 585 nm at pH 7.5. Catechol at 1 mM and at pH 7.5 rapidly destroyed the tyrosyl radical of *E. coli* R2, but had no effect on the iron centre (Figure 2). The limited loss of absorbance at 370 nm observed in Figure 2 is related to the reduction of the radical. It is actually well known that the tyrosyl radical has a small contribution to protein R2 spectrum at 370 nm [11]. When the reaction was carried out at alkaline pH and in the presence of higher concentrations of catechol, the 370 nm band, characteristic of the diferric site, gradually disappeared, while at the same time a new band centred at 490 nm appeared, indicating the formation of an iron-catecholate complex (inset to Figure 2). From the intensity of this band, one could calculate that approx. 3 iron atoms per molecule of R2 were mobilized by catechol. No band at 490 nm could be observed when catechol, at pH 8.8, was allowed to react with apoR2, the apoprotein form of R2 lacking both the iron centre and the radical.

The reaction of HSV protein R2 with 1 mM catechol, at pH 7.5, was found to result in the rapid and simultaneous reduction of the tyrosyl radical and the mobilization of the iron centre. This was shown from the parallel loss of the 370 nm band and the 410 nm peak and from the appearance of a broad absorption band at 585 nm, characteristic of the iron-catecholate complex (Figure 3).

DISCUSSION

E. coli protein R2 has been recently crystallized and its three-dimensional structure determined by X-ray diffraction [4]. It showed that the tyrosyl radical is deeply buried in the interior of the protein, at 1–2 nm (10–12 Å) from the closest protein surface. Moreover, the radical is located in a very hydrophobic pocket consisting of conserved phenylalanine and isoleucine residues. These structural characteristics explain why the reduction of the tyrosyl radical by hydroxamic acids, studied previously [6] and in the present paper, is mainly controlled by steric hindrance (Table 1). On the other hand, the very hydrophobic environment of the radical might explain why di- or tri-methylated phenols were found to be more active than phenol. The increased steric hindrance provided by the methyl groups in *o*- and *p*-positions might be compensated for by the increased reducing power and hydrophobic character of the compound. The presence of amino acids such as phenylalanine in the radical site may also explain why phenylhydroxamic acid was more active than ethylhydroxamic acid. Such an observation is consistent with recent results showing that phenylhydrazine was a better scavenger of the bacterial radical than were methylhydrazine or hydrazine [5].

Such studies may be useful for defining optimal structures for new anti-(ribonucleotide reductase) and thus anticancer or antiviral agents. However, all our results clearly show that the reactivity of the HSV radical greatly contrasts with that of the bacterial radical. First, the HSV tyrosyl radical is more reactive than the *E. coli* radical, suggesting it is less buried in the interior of the protein. Second, the HSV radical site allows less efficient

Scheme 1

hydrophobic interactions, in agreement with a more opened pocket. This suggests that the structures of the active sites are significantly different and consequently that data on *E. coli* enzyme cannot be extrapolated to the HSV enzyme.

No oxidation product has ever been identified during the reaction of a radical scavenger with protein R2. For example, how hydroxyurea is transformed during this reaction is still unknown, even though it is expected to react through a one-electron transfer to the radical. In the present study we demonstrate that, during reaction with R2, phenol is transformed into the corresponding diphenoquinone. The quinone was detectable by u.v.-visible spectroscopy only when methyl or methoxy groups were substituents on the *o*-positions of the phenol. This reaction is both tyrosyl-radical-dependent and oxygen-dependent. This shows that the reaction mechanism actually involves the transfer of one electron from the phenol compound to the protein radical and the intermediate formation of the corresponding phenoxyl radical. Such radicals are highly unstable and known to rapidly dimerize and oxidize in aerated solutions [8] (Scheme 1). A substitution at the *p*-position prevents the dimerization from occurring. This explains why no diphenoquinone could be observed during reaction of protein R2 with 2,4,6-trimethylphenol.

Finally, it is likely that chelation and mobilization of the iron centre of protein R2 might also participate in the inhibition of the enzyme activity. This mechanism has clearly to be considered in the case of HSV R2. There, iron can be easily mobilized by iron chelators such as catechol. Again, the difference with *E. coli* R2 is striking. The bacterial iron centre is tightly bound to the protein and much less reactive than the radical. Iron mobilization requires more drastic conditions: high concentrations of chelators and high pH. This again is consistent with a lower accessibility of the bacterial active site. That catechol removes iron from the

protein indicates that organic molecules may have physical access to the active site. Indeed, the formation of an iron catecholate complex is possible only if the chelator comes directly in contact with the ferric ions. This, at first sight, seems inconsistent with the rigidity of the protein and the inaccessibility of the iron centre, revealed by the three-dimensional structure of *E. coli* R2. Further studies are required before we can understand this aspect of protein R2 chemical reactivity.

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REFERENCES

- 1 Reichard, P. (1988) *Annu. Rev. Biochem.* **57**, 349–374
- 2 Fontecave, M., Nordlund, P., Eklund, H. and Reichard, P. (1992) *Adv. Enzymol.* **65**, 147–183
- 3 Eriksson, S. and Sberg, B.-M. (1989) *Allosteric Enzymes* (Herv, G., ed.), pp. 189–215. CRC Press, Boca Raton, FL
- 4 Nordlund, P., Eklund, H. and Sberg, B.-M. *Nature (London)* **345**, 593–598
- 5 Gerez, C. and Fontecave, M. (1992) *Biochemistry* **31**, 780–786
- 6 Kjller-Larsen, I., Sberg, B.-M. and Thelander, L. (1982) *Eur. J. Biochem.* **125**, 75–81
- 7 Mann, G. J., Grslund, A., Ochiai, E., Ingemarson, R. and Thelander, L. (1991) *Biochemistry* **30**, 1939–1947
- 8 Haynes, C. G., Turner, A. H. and Waters, W. A. (1956) *J. Chem. Soc.* 2823–2831
- 9 Battioni, J. P., Fontecave, M., Jaouen, M. and Mansuy, D. (1991) *Biochem. Biophys. Res. Commun.* **174**, 1103–1108
- 10 Sberg, B.-M., Hahne, S., Karlsson, M. H., Jrnvall, H., Gransson, M. and Uhlin, B. (1986) *J. Biol. Chem.* **261**, 2658–2662
- 11 Atkin, C. L., Thelander, L., Reichard, P. and Lang, G. (1973) *J. Biol. Chem.* **248**, 7464–7472
- 12 Barlow, T., Eliasson, R., Platz, A., Reichard, P. and Sberg, B.-M. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 1492–1495
- 13 Fontecave, M., Gerez, C., Mansuy, D. and Reichard, P. (1991) *J. Biol. Chem.* **265**, 10919–10924