Thiyl and phenoxyl free radicals and NADH

Direct observation of one-electron oxidation

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Absolute rate constants for the reaction of NADH with thiyl free radicals derived from various sulphur-containing compounds of biological significance were measured by using the technique of pulse radiolysis. These and related reactions with phenoxyl free radicals are believed to occur through one-electron-transfer processes. Further evidence comes from studies with deuterated NADH. The results support the possibility that, in biochemical systems, thiols may act as catalysts linking hydrogen-atom and electron-transfer reactions.

INTRODUCTION

Free radicals have now been implicated in normal physiological conditions as well as in many disease states (Pryor, 1976–83). Free-radical-mediated tissue injury, be it involving lipid, protein or nuclear material, can, in some cases, be modified on addition of an exogenous agent such as antioxidants or thiol-containing compounds (Porter & Whelan, 1983).

In the case of radiation-induced injury, thiol compounds in particular have been shown to be able to protect whole cells or animals from free-radical insult. A highly favoured hypothesis for such protection has been that of hydrogen transfer from the thiol to a free-radical-induced lesion (Prevot-Bernas, 1953; Wall & Magat, 1953; Howard-Flanders, 1960). If a lesion ($R^{\cdot}$) is formed through the oxidation of a critical molecule by a free-radical species under anoxic conditions it may be repaired by a thiol such as glutathione (GSH) according to:

In the absence of the protective agent it is supposed that the lesion $R^{\cdot}$ is a precursor of molecular damage. Such hydrogen-atom repair processes have been investigated in several systems by the use of e.s.r. (Ormerod & Alexander, 1963; Braams, 1963; Singh & Ormerod, 1965) and pulse radiolysis (Adams *et al.*, 1968, 1969; Baker *et al.*, 1982). Pulse radiolysis enables the reactions to be monitored directly, and the rate constants for the repair of simple carbon-centred radicals can be determined. For example, radicals derived from $OH^{\cdot}$ attack on simple alcohols have repair rates of approx. $10^8 \text{ M}^{-1} \cdot \text{s}^{-1}$ with the thiol cysteamine.

The possibility that phenol-containing compounds such as vitamin E (α -tocopherol) can act as antioxidants has also long been considered. In guarding against lipid peroxidation, for example, scavenging of propagating organic peroxy radicals and the formation of the tocopherol phenoxyl radicals are thought to be involved:

Clearly, as is the case with thiols, if protection is to be

effective the subsequent reactions of the resulting phenoxyl or thiyl radicals are crucial.

Thiyl free radicals have been viewed previously as relatively innocuous species undergoing some O_2 addition and hydrogen-atom-abstraction reactions as well as radical recombination. However, several reports have indicated that thiyl free radicals may themselves enter into one-electron-transfer reactions with a number of biomolecules, including ascorbate (Wolfenden & Willson, 1982; Asmus, 1983; Forni *et al.*, 1983; Forni & Willson, 1983; O'Neill, 1983). Phenoxyl radicals also undergo rapid electron-transfer reactions. Those from tyrosine and that from vitamin E, $\text{VitEO}^{\cdot}$, for example, have also been shown to react rapidly with ascorbate. The rate constants of electron transfer between substituted phenoxyl radicals have also been determined by pulse radiolysis and have allowed the determination of the one-electron redox potentials of these phenolate ions, in similar fashion to that described for quinones (Patel & Willson, 1973; Schuler, 1977; Packer *et al.*, 1979; Steenken, 1979; Steenken & Neta, 1979, 1982).

We now present evidence for electron-transfer reactions involving thiyl and phenoxyl free radicals and NADH. The results support the concept that there may be situations where through coupled hydrogen-atom- and electron-transfer reactions potentially damaging lesions caused by free-radical attack may be repaired by normal cellular processes:

MATERIALS

All chemicals used were of the purest grade available and were used without further purification, unless otherwise stated. Acetone, ethanol, 2-methylpropan-2-ol (subsequently purified by fractional crystallization), propan-2-ol, KSCN and NaN_3 (AnalaR) were supplied by BDH Chemicals. Cysteamine, penicillamine, GSH,

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GSSG, cysteine (hydrochloride), tyrosine, NAD⁺, NADH, 5,5'-dithiobis-(2-nitrobenzoic acid), methyl viologen, tryptophyltyrosine and yeast alcohol dehydrogenase (specific activity 450 units/mg) were obtained from Sigma Chemical Co. Thiola [N-(2-mercaptopropionyl)glycine] was a gift from Santen Pharmaceuticals. Deuterated ethanol, Trolox C, phenol, *m*-cresol and 2,6-dimethylphenol were obtained from Aldrich Chemical Co. All gases were supplied by the British Oxygen Co.

METHODS

Radiolysis experiments were performed with the Brunel University radiation facilities. The pulse-radiolysis experiments were undertaken with the 4MeV linear accelerator with a 200 ns electron pulse (Willson, 1978, 1982). Doses of up to 15 J·kg⁻¹ were used as determined by thiocyanate dosimetry (Adams *et al.*, 1965). All solutions were prepared just before experimentation in Millipore-filtered distilled water. Solutions for pulse radiolysis were saturated with N₂O or N₂ by using the syringe bubbling technique (Willson, 1970b), and the pH was adjusted where necessary with NaOH or HCl. All solutions were exposed to the minimum of light before irradiation.

Stationary-state radiolysis studies were undertaken with the Brunel 2000 Ci 'cave-type' ⁶⁰Co radiation source, at a dose rate of 19 J·kg⁻¹·min⁻¹ (Gy·min⁻¹). Samples were irradiated in borosilicate tubes. During long irradiations the samples were rotated at regular intervals in order to obtain a uniform dose within the sample. Dosimetry was performed with the Fricke dosimeter, taking $G(\text{Fe}^{3+}) = 15.6 \text{ mol} \cdot \text{J}^{-1}$ (Fricke & Hart, 1966). The doses used experimentally ranged from 100 to 500 J·kg⁻¹.

Thiyl free radicals were generated in two ways. In the pulse-radiolysis studies de-aerated aqueous solutions (pH 6–7) containing the thiol (typically 50 mM), propan-2-ol and acetone (1 M) were used. Under these conditions the following processes predominate:

For the γ -radiolysis experiments, glutathione thiyl (GS[•]) radicals were generated from the disulphide GSSG (10 mM) in N₂-saturated systems (pH 7) containing 1 M-2-methylpropan-2-ol according to:

Thiol concentrations were determined with Ellman's reagent, 5,5'-dithiobis-(2-nitrobenzoic acid), which

Fig. 1. Absorption observed at 340 nm after pulse radiolysis of an N₂-saturated solution containing 1 M-acetone/propan-2-ol, 50 mM-GSH and 80 μM-NADH at pH 6

undergoes a thiolate disulphide exchange reaction to yield 2-nitromercaptobenzoic acid (Ellman, 1959). This was measured spectrophotometrically against an appropriate blank, with a Varian Cary 219 spectrophotometer.

Phenoxyl radicals can be generated under pulse-radiolysis conditions through the reaction of a variety of species, including the inorganic radicals Br₂^{•-} and N₃[•], which themselves can be generated in N₂O-saturated solutions of excess bromide or azide. Rapid one-electron oxidation of the phenolic compounds generating the corresponding phenoxyl radicals are thought to occur (Hayon & Simic, 1970; Prutz & Land, 1978; Butler *et al.*, 1984):

At pH 11.8 the reaction between tyrosine and N₃[•] proceeds at a rate of $3.6 \times 10^8 \text{ M}^{-1} \cdot \text{s}^{-1}$ whereas at pH 6.5 the rate is decreased to $1 \times 10^8 \text{ M}^{-1} \cdot \text{s}^{-1}$ (Land & Prutz, 1979).

Deuterated NADH (NAD²H) was prepared enzymically from NAD⁺ and [²H₆]ethanol by using yeast alcohol dehydrogenase (Rafter & Colowick, 1957). A 1 g portion of NAD⁺ was added to 100 ml of 0.5 M-Tris/HCl buffer, pH 7.0, containing 0.5 M-[²H₆]ethanol and incubated at 25 °C. On equilibration, 5 mg of yeast alcohol dehydrogenase was added. After a 45 min incubation the percentage conversion into NAD²H was assessed spectrophotometrically at 340 nm. The reaction was stopped by addition of 25% (w/v) barium acetate followed by 50 ml of industrial ethanol. The NAD²H was collected after incubation at 0 °C in industrial ethanol for 2 h by fractional centrifugation. The isotopic purity of the NAD²H was checked by both n.m.r. and mass spectrometry. Mass-spectral evaluation of the samples was performed (at University College London by Dr. I. C. Shaw and Dr. M. Mruzek) with a Finnigan Incos 2300 data system and confirmed the presence of

Fig. 2. Change in absorption spectra monitored 400 μ s after pulse radiolysis of an N_2 -saturated solution containing 1 M-acetone/propan-2-ol, 10 mM-cysteine and 80 μ M-NADH at pH 7

The dose was 19 Gy.

deuterium in the NAD^2H sample. Control samples of NADH were prepared from NAD^+ and ethanol in an identical manner.

RESULTS AND DISCUSSION

Reactions of thiol radicals with NADH

On pulse radiolysis of an N_2 -saturated solution containing 1 M-acetone/propan-2-ol, 1 mM-GSH and 80 μ M-NADH, at pH 6, a rapid decrease in light-absorption was observed at 340 nm. Omission of the thiol did not result in such a decrease (Fig. 1). Spectral analysis of this and related systems containing thiols showed that the decrease in absorption had a maximum at 340 nm, characteristic of NADH. A small increase in absorption at approx. 400 nm was also apparent (Fig. 2). The results were tentatively ascribed to reactions such as:

The absorption at approx. 400 nm was similar to that observed on oxidation of NADH by $Br_2^{\bullet-}$ and previously assigned to the species $NAD^\bullet$ (Land & Swallow, 1970). On addition of O_2 to the system the absorption at 400 nm decayed rapidly, in agreement with $k_{(22)} = 1.9 \times 10^9 \text{ M}^{-1} \cdot \text{s}^{-1}$ (Willson, 1970a):

To confirm that the proposed reaction (21) does lead to $NAD^\bullet$ formation, solutions with methyl viologen added were examined. On pulse radiolysis of an N_2 -saturated solution containing 1 M-acetone/propan-2-ol and

Fig. 3. Transient absorption spectra observed after pulse radiolysis of an N_2 -saturated solution containing 1 M-acetone/propan-2-ol and 0.1 mM-methyl viologen alone (●), plus 10 mM-GSH (○) or plus 10 mM-GSH and 1 mM-NADH (□) at pH 6

Fig. 4. Yield of thiol as measured by Ellman's reagent after radiolysis of N_2 -saturated solutions containing 1 M-2-methylpropan-2-ol and 10 mM-GSSG (○) and 1 mM-NADH (●) at pH 7

0.1 mM-methyl viologen (MV^{2+}) at pH 6 the transient absorption shown in Fig. 3, characteristic of the methyl viologen radical cation, was observed, in agreement with $k_{(23)} = 3.0 \times 10^9 \text{ M}^{-1} \cdot \text{s}^{-1}$ (Farrington *et al.*, 1973):

On addition of 10 mM-GSH to the system (a concentration sufficient to scavenge more than 95% of all the propan-2-ol radicals formed), the $MV^{\bullet+}$ absorption was decreased to less than 8% of that found in the absence of the thiol (Fig. 3). Addition of 1 mM-NADH to the system restored the $MV^{\bullet+}$ absorption, in agreement with

Fig. 5. Plot of first-order rate constant for the decrease in absorption at 340 nm at pH 6 after pulse radiolysis of N_2 -saturated solutions containing 1 M-acetone/propan-2-ol, 50 mM-GSH and NADH (●) or NAD^2H (○)

reactions (10) and (21) followed by (24) (Farrington *et al.*, 1980; Anderson, 1980):

Confirmation that either thiolate ions or free thiol are formed in reaction (21) was obtained from stationary-state γ -radiation studies in which glutathione thiyl radicals were generated by reduction of GSSG (reactions 12–15). On γ -radiolysis of N_2 -saturated aqueous solutions of GSSG (10 mM) containing 2-methylpropan-2-ol the yield of free thiol produced, measured with Ellman's reagent, was found to be $0.3 \mu\text{mol} \cdot \text{J}^{-1}$ (Fig. 4). This formation is attributed to the reactions (12)–(16). Under these conditions the yield of e^-_{aq} and H^+ is known to be approx. $0.33 \mu\text{mol} \cdot \text{J}^{-1}$ ($G = 3.3$), in close agreement with the yield of GSH obtained. When similar experiments were performed in the presence of 1 mM-NADH the yield of GSH determined was $0.65 \text{ mol} \cdot \text{J}^{-1}$ ($G = 6.5$), in agreement with reaction (21) followed by reaction (16).

Clearly these results demonstrate that thiyl radicals react rapidly with NADH with the net formation of GS^- or GSH. However, they do not provide any direct evidence as to the mechanism in favour of either an initial electron-transfer reaction (25) or a hydrogen-atom-transfer process (26):

NADH is known to undergo rapid one-electron-transfer reactions with the free radical anions $Br_2^{\cdot-}$ and $(SCN)_2^{\cdot-}$, the mechanism proposed being (Land & Swallow, 1970):

Table 1. First-order bimolecular rate constants for the reaction between thiyl radicals and NADH

The percentage yields are calculated assuming a thiyl radical yield of $0.6 \mu\text{mol} \cdot \text{J}^{-1}$ in 1 M-acetone/propan-2-ol and $\epsilon_{340} = 6220 \text{ M}^{-1} \cdot \text{cm}^{-1}$.

Thiol	$10^{-8} \times k \text{ (M}^{-1} \cdot \text{s}^{-1})$	Yield (%)
Cysteamine	23 ± 0.5	90
Cysteine	5.8 ± 0.3	78
Thiola	4.1 ± 0.2	70
Penicillamine	3.1 ± 0.3	65
GSH	2.3 ± 0.2	70
GSH	$2.3 \pm 0.2^*$	70

* Rate obtained with deuterated coenzyme.

where $X_2^{\cdot-}$ represents $Br_2^{\cdot-}$ or $(SCN)_2^{\cdot-}$. By analogy the reaction between the thiyl radicals and NADH may be expressed as:

Although NADH has been reported to react, albeit inefficiently, with carbon-centred radicals (Chan & Bielski, 1975), the fact that no decrease in absorption at 340 nm was observed in the absence of thiol (indicating $k_{(30)} < 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$) suggests that it does not readily enter into hydrogen-transfer reactions:

Further support for an electron-transfer mechanism was provided by studies with deuterated NADH, i.e. NAD^2H .

The plots of first-order rate constant against the concentration of NADH and NAD^2H for the reaction of the glutathione thiyl radical in N_2 -saturated aqueous solutions of 1 M-acetone/propan-2-ol at pH 6 are shown (Fig. 5). No appreciable difference between the rate constant determined for the reaction between $GS^{\cdot}$ and either NADH or NAD^2H is apparent, i.e. k_H/k_D is unity (Table 1). The implication is therefore that the reaction does not involve hydrogen-atom or deuterium-atom transfer but proceeds through a one-electron-transfer mechanism, i.e. reaction (25) followed by reaction (28).

When GSH was replaced by the thiols cysteamine, cysteine, *N*-(2-mercaptopropionyl)glycine and penicillamine similar results were obtained (Fig. 6). The first-order bimolecular rate constants are given (Table 1). The yields of NADH depletion following thiyl radical attack have been calculated from measurements of the loss of absorption at 340 nm (Table 1). In all the studies the full yields of thiyl radicals expected if they were reacting with the NADH stoichiometrically were not obtained. This may be explained either by the thiyl radicals reacting at sites other than the nicotinamide ring, or, more likely, by the scavenging of the radicals by thiolate anions. The concentration of thiol used was 10 mM, and therefore at pH 6 in the case of cysteine or cysteamine there would have been approx. 5 mM thiolate anion present. Given that the rate for the reaction:

is $k_{(31)} = 4.9 \times 10^9$ and $3.1 \times 10^9 \text{ M}^{-1} \cdot \text{s}^{-1}$ for cysteamine and cysteine respectively, the theoretical percentage of

Fig. 6. Plot of first-order rate constant for the decrease in absorption at 340 nm at pH 6 after pulse radiolysis of N_2O -saturated solutions containing 1 M-acetone/propan-2-ol, NADH and 50 mM-cysteamine (●) or 50 mM-cysteine (■) or 50 mM-GSH (○)

the thiyl radicals reacting with NADH is calculated to be 87% for cysteamine and 77% for cysteine. This compared favourably with the yields of 90% and 78% achieved experimentally. The rates of reaction obtained (Table 1) are extremely high, particularly when compared with those rate constants published for hydrogen-atom abstractions from organic compounds by thiyl radicals, or of hydrogen-atom transfer from NADH (Walling & Rabinowitz, 1959; Pryor *et al.*, 1973; Miyashita *et al.*, 1977). For example, for the reaction:

$k_{(32)} = 1.8 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$ has been obtained, considerably lower than the rate constant observed here (Nadezhidin & Dunford, 1979).

Hydrogen-atom abstraction by the penicillamine thiyl radical has also been observed by pulse radiolysis:

The reaction is again relatively slow, with $k_{(28)} = 3.8 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$, where $\text{RS}^{\cdot}$ is the penicillamine thiyl radical (Schafer & Asmus, 1981). There have been several reports of one-electron transfer from NADH to relatively strong oxidants; for example, NADH model compounds are rapidly oxidized by ferricyanide (Okamoto *et al.*, 1977), and it appears that one-electron oxidants with potentials greater than 600 mV do undergo one-electron-transfer reactions with NADH. Given that theoretical considerations have indicated that the thiyl radical redox couple $\text{RS}^{\cdot}/\text{RS}^-$ has a one-electron potential of approx. 1.0 V, this provides further support for an electron-transfer mechanism (Ahmad & Armstrong, 1984).

Reactions of phenoxyl radicals with NADH

On pulse radiolysis of N_2O -saturated solutions containing 0.1 M- NaN_3 and 5 mM-tyrosine at pH 7, a transient absorption was observed that decayed very slowly, with a first half-life of 0.2 ms. On addition of 18 μM -NADH to the solution, however, there was a rapid decay in the $\text{TyrO}^{\cdot}$ absorption (Fig. 7a) and a concomitant bleaching at 340 nm (Fig. 7b), in agreement with the oxidation of NADH by $\text{TyrO}^{\cdot}$. Azide radicals are known to oxidize NADH directly: $k =$

Fig. 7. (a) Transient absorption observed at 410 nm after pulse radiolysis of an N_2O -saturated solution at pH 7 containing 0.1 M- NaN_3 , 5 mM-tyrosine and 18 μM -NADH, and (b) the absorption observed on pulse radiolysis of an identical solution at 340 nm

$3 \times 10^8 \text{ M}^{-1} \cdot \text{s}^{-1}$ at pH 7 (L. G. Forni & R. L. Willson, unpublished work). The concentration of NADH was therefore maintained at a level where at least 90% of the azide radicals would react with the tyrosine directly. At both 410 and 340 nm the decrease in absorption was exponential with time, the rate of decay increasing with increasing NADH concentration. The resulting kinetic plot (Fig. 8) corresponds to $k_{(34)} = 6.1 \times 10^7 \pm 0.5 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$:

When tyrosine was replaced by phenol or *m*-cresol, similar observations were obtained. However, no reaction between the related Trolox C or 2,6-dimethylphenoxyl free radicals was observed. The rate constants obtained are shown (Table 2).

Tyrosine phenoxyl radicals can also be formed in tryptophyltyrosyl dipeptides after selective one-electron oxidation of the tryptophan by $\text{N}_3^{\cdot}$ (Prutz & Land, 1979; Prutz *et al.*, 1980). On pulse radiolysis of an N_2O -saturated solution at pH 7 containing 50 mM- NaN_3 , 3 mM-tryptophyltyrosine (HTrp-TyrOH) and 25 μM -NADH the characteristic absorption spectrum of $\text{Trp}^{\cdot}$ was found after 5 μs , in agreement with:

After 25 μs the $\text{Trp}^{\cdot}$ absorption spectrum was replaced by that of $\text{TyrO}^{\cdot}$, in agreement with the subsequent reaction:

However, after 3 ms the absorption due to $\text{TyrO}^{\cdot}$ decayed, leaving a bleaching at 340 nm as described above. The rate constant derived from observations of the decay of the tyrosine phenoxyl radical or the bleaching of the NADH was $k = 5.5 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$ with

Fig. 8. Plot of first-order rate constant for the decrease in absorption at 340 nm (●) and at 410 nm (○) after pulse radiolysis of N_2O -saturated solutions at pH 7 containing 0.1 M-Na $_2$ S, 5 mM-tyrosine and NADH

Table 2. First-order bimolecular rate constants for the reaction between phenoxyl radicals and NADH

Phenol	$10^{-7} \times k \text{ (M}^{-1} \cdot \text{s}^{-1}\text{)}$
Tyrosine	6.1 ± 0.5
Phenol	8.0 ± 0.2
<i>m</i> -Cresol	2.7 ± 0.2
2,6-Dimethylphenol	$< 10^6$
Trolox C	$< 10^6$

a yield of NADH destruction of approx. 70%. This compares favourably with the results determined above for that of the tyrosine phenoxyl radical alone.

The observation that phenoxyl radicals can oxidize NADH is particularly interesting with respect to protein damage by free-radical species. Phenoxyl radicals are known to react rapidly with a variety of electron donors, including ascorbate and various ionized phenols (Schuler, 1977; Packer *et al.*, 1979; Steenken & Neta, 1979, 1982). By analogy, the mechanism proposed for the reaction of $RO^\bullet$ with NADH is again one of direct electron transfer between the phenoxyl radical and NADH via:

In the case of tyrosine, recent work has shown that the tyrosyl radical, $TyrO^\bullet$, may rapidly oxidize the electron donors metiazinic acid and 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonate) (ABTS) (L. G. Forni & R. L. Willson, unpublished work).

$$k_{(38)} = 1.2 \times 10^9 \pm 0.1 \times 10^9 \text{ M}^{-1} \cdot \text{s}^{-1}$$

Similar reactions may also be important in the mode of action of some enzymes. For example, an intermediate in the action of ribonucleotide reductase possesses a tyrosine free radical at its active site (Graslund *et al.*, 1982).

CONCLUSIONS

Clearly the present results and previous experiments strongly indicate that both thiyl and phenoxyl radicals are able to enter into one-electron-transfer reactions. Thiols such as GSH may thus act as catalysts repairing free-radical-induced lesions by redox cycling processes involving both hydrogen-atom- and electron-transfer reactions.

Clearly, before an estimate can be made of the relative importance of such reactions *in vivo*, a knowledge of the nature of any critical radicals $R^\bullet$, the rate constants of the above reactions and the local concentrations of NADH, RSH and O_2 will be required.

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