COMMUNICATION

The catalysed NADH reduction of resazurin to resorufin

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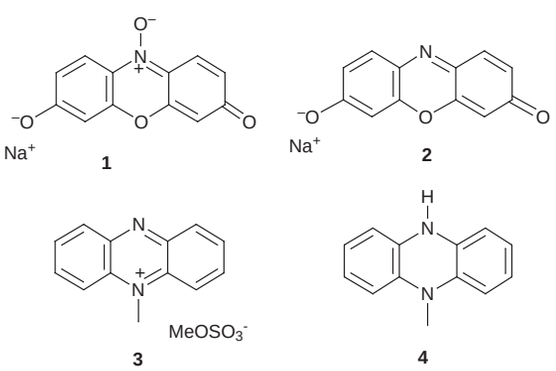
Received (in Cambridge) 14th August 1998, Accepted 28th September 1998

Details are reported on the mechanism whereby NADH can be used for the reduction of resazurin 1 to give the fluorescent product resorufin 2, a process requiring the use of a catalyst, such as *N*-methylphenazinium methosulfate 3.

NADH and NAD(PH) are important biochemical vectors and measurement of intracellular turnover of these reagents is often used to indicate cellular viability and activity.[1] NAD(P)H concentrations can be monitored by direct fluorescence but, since it is rapidly turned over [re-oxidised to $NAD(P)^+$], absolute levels do not necessarily reflect the level of enzymic and metabolic activity occurring within the cell. As a consequence there is considerable interest in using reagents that themselves are selectively reduced by NAD(P)H with the formation of either a coloured or fluorescent signal. Few dye precursors react directly with NAD(P)H and, in order to overcome this problem, many chemists have shown that certain enzymes, such as diaphorase,[2] can act as electron carriers in order to effect reduction. Thus, the reduction of tetrazolium dyes, such as iodonitrotetrazolium blue, by NADH is catalysed by diaphorase, to form the intensely violet formazan dye. However the product formazans are not fluorescent.[3] Use of the dye resazurin **1**, which, when pure, is only weakly fluorescent, has also been made and diaphorase also catalyses the reduction of this dye by NADH to the strongly fluorescent product, resorufin, **2**.[4]

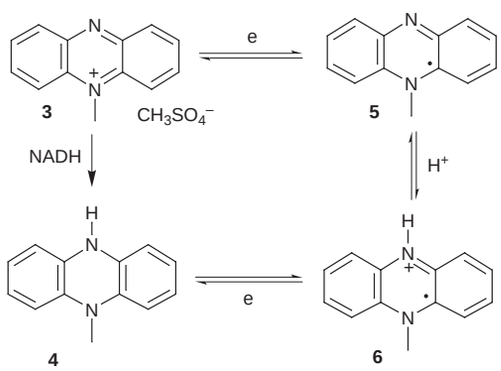


The details of the mechanism whereby the enzyme diaphorase acts as a catalyst do not seem to have been investigated. We report here some progress with the use of the organic catalyst *N*-methylphenazinium methosulfate (PMS^+, **3**) as an alternative to the use of enzymes for catalyzing the reduction of resazurin. PMS^+ has been widely used as an electron transfer agent in a range of enzyme assays and potentiometric titrations.[5]

Mixing NADH with resazurin **1** in a buffer at pH 5–8 gave no reaction and *no* formation of the fluorescent reduction product, resorufin **2**, occurred. When the experiment was repeated in the presence of PMS^+ **3**, reduction of the resazurin **1** occurred to form the highly fluorescent dye, resorufin **2**. This change was pH dependent, the highest conversion of resazurin to resorufin occurring at pH 6.5. The overall reduction reaction is shown in eqn. (1).

$$\text{NADH} + \mathbf{1} + \text{H}^+ \xrightarrow[\text{catalyst}]{} \text{NAD}^+ + \mathbf{2} + \text{H}_2\text{O} \qquad (1)$$

The mechanism of this catalysis has been studied. When NADH and PMS^+ **3** are mixed, a rapid reaction occurs in which NADH disappears, to form NAD^+, with concomitant formation of the unstable hydrophenazine derivative **4**. Since NADH mainly acts as a two-electron reductant,[6] it is assumed that, in this reaction, the hydrophenazine **4** is formed directly. In our studies, no sign of any long-lived intermediates such as **5** and **6** (Scheme 1) could be observed in the reaction between



Scheme 1

3 and NADH. At neutral pH, compound **4** is only sparingly soluble in water but, although it can be isolated by extraction into dichloromethane, the product is unstable to air, highly coloured products forming.[7] The hydrophenazine can also be formed by careful reduction of PMS^+ with sodium borohydride. Although the hydrophenazine **4** is formally 'anti-aromatic' and expected to be a good reducing agent, by itself it was found *not* to reduce resazurin, even after several hours, so it is *not* acting as the catalytic species. Another, phenazinium-derived, species must therefore be responsible for the catalytic action.

Pulse radiolysis studies were carried out in order to gain some insight into the overall reduction processes. Radiolysis of aqueous sodium formate solutions (0.1 M) saturated with nitrous oxide yields the formate radical anion ($CO_2^{\bullet -}$), a one-electron donor. This species rapidly reduced PMS^+ **3**, to give a new species, the reduced product $PMS^{\bullet}$, **5** (Scheme 1). Different spectra were obtained at pH 5 and pH 9 indicating that this radical can be protonated, to give another new species, $PMSH^{\bullet +}$, **6**, assigned to the equilibrium shown in eqn. (2).

$$\underset{\mathbf{5}}{\text{PMS}^{\bullet}} + \text{H}^+ \rightleftharpoons \underset{\mathbf{6}}{\text{PMSH}^{\bullet +}} \qquad (2)$$

Under acidic conditions the species **6** is stable for periods of hours and is unaffected by the presence of starting PMS^+, **3**. However, in alkaline solution (pH 9.5) it was found to undergo a *pseudo*-first order reaction in which the rate was dependent on the concentration of the parent compound (PMS^+), $k_{obs} \approx 7 \times 10^3$ s^{-1} at $[PMS^+] = 100$ μM and $k_{obs} \approx 3 \times 10^4$ s^{-1} at $[PMS^+] = 1$ mM. At the higher concentration a new absorption band at *ca.* 710 nm was observed, suggesting the formation of a complex **7** between $PMS^\bullet$ **5** and the parent PMS^+ **3** [eqn. (3)].

$$\underset{\mathbf{5}}{PMS^\bullet} + \underset{\mathbf{3}}{PMS^+} \rightleftharpoons \underset{\mathbf{7}}{[PMS^\bullet \cdot PMS^+]} \qquad (3)$$

The species **5** shows absorbance at 580 nm and the pK_a of the species $PMSH^{\bullet+}$ was determined by monitoring this absorbance at different pH *before* the formation of the complex **7**, to yield the value p$K_a = 7.72 \pm 0.04$ (at room temperature and ionic strength ≈ 0.1 M). The equilibria (2) and (3) lead to the prediction that the apparent pK_a value measured after formation of the complex (as would be obtained by standard methods) should be dependent on the concentration. This would explain the conflicting pK_a values of 6.8[8] and 5.7[9] reported for the species **6** in the literature.

Under the conditions used for the reduction reaction, PMS^+ **3** and PMSH **4** interact to form the active species $PMS^\bullet$ **5**, according to eqn. (4). Resazurin **1** was then subjected to

$$\underset{\mathbf{3}}{PMS^+} + \underset{\mathbf{4}}{PMSH} \longrightarrow 2\underset{\mathbf{5}}{PMS^\bullet} + H^+ \qquad (4)$$

reduction under pulse radiolysis conditions, using the formate anion, $CO_2^{\bullet-}$. A rapid one-electron reduction occurs. The reduction was followed by bleaching of the resazurin absorption peak at 630 nm followed by a partial recovery of the absorbance, which followed second order kinetics. This is assigned to the reduction of **1** to the respective radical anion, $\mathbf{1}^{\bullet-}$ followed by its disproportionation[10] to yield resazurin and resorufin, **2** [eqns. (5) and (6)]. At pH 5, addition of PMS^+ **3** to

$$\mathbf{1} + CO_2^{\bullet-} \longrightarrow \mathbf{1}^{\bullet-} + CO_2 \qquad (5)$$

$$2\,\mathbf{1}^{\bullet-} + 2\,H^+ \longrightarrow \mathbf{1} + \mathbf{2} + H_2O \qquad (6)$$

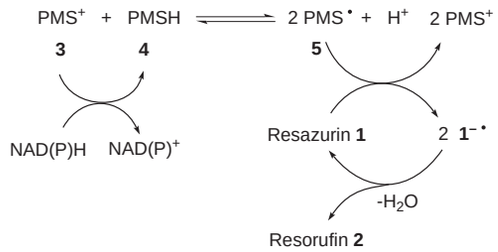
the intermediate species $\mathbf{1}^{\bullet-}$ leads to formation of $PMSH^{\bullet+}$ **6** and resazurin **1**, indicating that the stable species $PMSH^{\bullet+}$ is, thermodynamically, not able to reduce resazurin. However, when $PMSH^{\bullet+}$, generated radiolytically at pH 5, is added to an excess of resazurin a relatively slow reaction occurs leading to the formation of resorufin. The disproportionation, reaction (6), is therefore an important step in helping to drive the overall process forward.

The role of PMS^+ as a catalyst for the reduction of resazurin must therefore be in a kinetically controlled process involving the equilibria (2) and (4). The $PMS^\bullet$ radical **5** can then react with resazurin **1** to form a small quantity of the radical anion, $\mathbf{1}^{\bullet-}$, which then undergoes the irreversible process (6), yielding the fluorescent product resorufin, **2**.

Since the active, reduced phenazinium species **5** can also react with oxygen and the phenazinium cation is light sensitive,[7] the detection of NAD(P)H is best carried out in the dark in the presence of a relatively large amount of the phenazinium catalyst. Under these conditions, the reproducible formation of the fluorescent resorufin is observed.

Although the radical species generated in these reactions can interact with oxygen producing, *inter alia*, the superoxide anion,[11] we have found that the addition of superoxide ion to resazurin does *not* produce resorufin, so this species is also not involved in the reduction. This is in contrast to the known reduction of tetrazolium salts to formazans effected by superoxide ions.[12]

The overall reduction process is summarised in Scheme 2,



Scheme 2

from which it can be seen that the role of the catalyst is to act as a one-electron carrier to resazurin.

The detection of NADH [or NAD(P)H] formation and turnover by the catalysed formation of the fluorescent product resorufin is currently being investigated as a simple means for enhancing the widely-used but error-prone Papanicolau test for the early detection of cervical cancer.[13]

Experimental

NADH, as the disodium salt and phenazine were purchased from Sigma-Aldrich Co. Ltd, Poole, Dorset. The phenazine was methylated with redistilled dimethyl sulfate to produce the yellow phenazinium methosulfate, mp 158–160 °C (decomp.) using a modified literature method.[14] Solutions were freshly prepared each day and stored in the dark before use. The principal buffer used was *N′*-(2-hydroxyethyl)piperazine-*N*-ethanesulfonic acid (HEPES), adjusting the pH with 0.1 mol dm^{-3} sodium hydroxide or hydrochloric acid. The γ-radiolysis studies were conducted using the pulse radiolysis facilities of the Gray Laboratory, using a ^{60}Co source with a nominal activity of 2000 Ci. Fluorescence measurements were made on a Perkin-Elmer LS50B luminescence spectrometer and resorufin fluorescence was examined using λ_{ex} 545 nm and λ_{em} 583 nm.

Acknowledgements

We thank the EPSRC for a research studentship (to C. A. R.) and a research grant (N. L. M.).

References

1 R. P. Haughland, *Handbook of Fluorescent Probes and Research Chemicals*, Molecular Probes Inc., Eugene, Oregon, USA, 6th edn., 1997, ch. 16.3, pp. 381–382; J. T. Mosmann, *Immunol. Methods*, 1983, **65**, 55; H. Imamura, S. Takao and T. Aikou, *Cancer Res.*, 1994, **54**, 3620; S. W. Thom, R. W. Horobin, E. Seidler and M. R. Barer, *J. Appl. Bacteriol.*, 1993, **74**, 433.
2 C. H. Self, *J. Immunol. Methods*, 1985, **76**, 389.
3 E. Seidler, *The Tetrazolium-Formazan System: Design and Histochemistry*, in *Progress in Histochemistry and Cytochemistry*, Gustav Fischer Verlag, Stuttgart, 1991, vol. 24, pp. 1–86.
4 D. B. Cook and C. H. Self, *Clin. Chem.*, 1993, **39**, 965.
5 *Cf.* F. G. Halaka, G. T. Babcock and J. L. Dye, *J. Biol. Chem.*, 1982, **257**, 1458.
6 G. Blankenhorn, in *Pyridine Nucleotide Dependent Dehydrogenases*, ed. H. Sund, W. de Gruyter & Co., Berlin, 1977, pp. 185–205.
7 G. Davis and P. J. Thornalley, *Biochem. Biophys. Acta*, 1983, **724**, 456.
8 P. S. Rao and E. Hayon, *Anal. Chem.*, 1976, **48**, 564.
9 W. Rubaszewska and Z. R. Grabowski, *J. Chem. Soc., Perkin Trans. 2*, 1975, 417.
10 W. Prutz, J. Butler and E. Land, *Arch. Biochem. Biophys.*, 1996, **327**, 239.
11 A. K. Raap, *Histochem. J.*, 1983, **15**, 977.
12 M. Nishikimi, M. N. A. Rao and K. Yagi, *Biochem. Biophys. Res. Commun.*, 1972, **46**, 849.
13 V. Ponti, M. V. Damiani, K. Cheeseman and T. F. Slater, *Chem. Biol. Interact.*, 1978, **23**, 281.
14 F. Kehrmann and E. Havas, *Ber.*, 1913, **46**, 341.

Communication 8/06431H