Investigation of the arylnitroso reductase activity of pig liver aldehyde reductase

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The reduction of *p*-nitroso-*N*-dimethylaniline, *p*-nitroso-*N*-diethylaniline, *p*-nitrosophenol and *p*-nitroso-*N*-phenylaniline with NADPH in the presence of aldehyde reductases 1 and 2 is described. The reactivity of these nitroso substrates is increased by hydrophobic substituents and those promoting OH[−] elimination from the molecule of the reduced substrate. *NN*-Dimethylbenzoquinonedi-iminium cation was proved to be the reaction product formed from *p*-nitroso-*N*-dimethylaniline. The kinetics of the reduction of *p*-nitroso-*N*-dimethylaniline catalysed with aldehyde reductase 1 are rather complex at pH 7, and the preferred-pathway mechanism is probably involved. The reaction sequence approaches the ordered pattern at pH 8.5. It was shown that NADPH ⇌ NADP⁺ recyclization proceeds in the presence of NADP⁺, *p*-nitroso-*N*-dimethylaniline, cyclohexanol and aldehyde reductase 1, the alcohol oxidation being the slowest step in this reaction. However, the rate of cyclohexanol oxidation surpasses that of the dissociation of NADPH from the enzyme.

INTRODUCTION

Monomeric NADPH-dependent aldehyde reductases (EC 1.1.1.2) are enzymes with a broad substrate specificity [1, 2]. They are able to reduce a variety of aldehydes, aromatic compounds being especially preferred [2]. Aldehyde reductases 1 and 2 from pig liver are typical representatives of the family of mammalian aldehyde reductases [3]. Aldehyde reductase 1 seems to be identical with or very similar to aldehyde reductase from pig kidney [2]. The kinetics of these enzymes obey the ordered mechanism for most substrates [3, 4]; however, more complex mechanisms are thought to occur in some cases [5, 6]. There is a possibility with an enzyme, such as aldehyde reductase, that uses a large number of substrates that the reaction mechanism may vary.

In the present paper we describe the results of kinetic experiments with novel analogues of substrates of aldehyde reductase. Arylnitroso compounds, which have structures isosteric and isoelectronic with aldehydes, are known to be excellent substrates of NAD⁺/NADH-dependent animal alcohol dehydrogenases (which have a substrate specificity comparable with that of aldehyde reductases) [7–9]. Our investigation is aimed at description of the reduction of these compounds in the presence of NADPH and aldehyde reductases and at analysis of both kinetic and chemical mechanisms of these reactions.

MATERIALS AND METHODS

Materials

p-Nitroso-*N*-dimethylaniline, *p*-nitroso-*N*-diethylaniline, *p*-nitrosophenol, *p*-nitroso-*N*-phenylaniline and nitrosobenzene were prepared and kindly donated by Dr. L. Skurský and Dr. P. Karlovský. These substances were synthesized in accordance with refs. [10–12]. NADPH (Reanal, Budapest, Hungary) was purified chromatographically as described previously [13]. NADP⁺ was the product of Boehringer (Mannheim,

Germany). Cyclohexanol and *p*-nitrobenzaldehyde were supplied from Fluka (Buchs, Switzerland). Salicylamide was purchased from BDH Chemicals (Poole, Dorset, U.K.). Aldehyde reductases (homogeneous aldehyde reductase 1 and partially purified aldehyde reductase 2) were isolated and assayed as described previously [13].

Initial-rate measurements

These were performed spectrophotometrically (Cary 118 instrument) either at 340 nm (changes in NADPH concentrations) or at 440 nm (changes in the concentrations of arylnitroso compounds, which have molar absorption coefficients in the range 33000–35000 M^{−1}·cm^{−1}; ref. [8]). The reactions were started with the addition of enzyme. The experiments were carried out in triplicate in 0.1 M-sodium phosphate buffer, pH 7.0, or 0.1 M-sodium pyrophosphate buffer, pH 8.5, at 25 °C. For presentation purposes the kinetic data are shown in double-reciprocal plots.

$$[E]/v_0 = \phi_0 + \phi_1/[S_1] + \phi_2/[S_2] + \phi_{12}/([S_1] \cdot [S_2]) \quad (1)$$

In eqn. (1) $[E]$ is the concentration of enzyme active sites (determined as described previously [4, 13]), $[S_1]$ and $[S_2]$ are the concentration of the coenzyme (NADPH) and substrate (*p*-nitroso-*N*-dimethylaniline) respectively (or of cyclohexanol and *p*-nitroso-*N*-dimethylaniline in the recyclization reactions measured at the excess of NADP⁺). ϕ_1 , ϕ_2 , ϕ_0 and ϕ_{12} stand for the kinetic parameters that are the functions of the underlying rate constants [14]. The values of these kinetic coefficients were computed by non-linear regression [15]. The data obtained in the presence of NADPH and *p*-nitroso-*N*-dimethylaniline at pH 7 were subjected to an error analysis [15]. The variance (σ^2) was found to obey the proposed formula [16, 17]:

$$\sigma^2 = \sigma_0^2 + \sigma_2^2 \cdot v_0 \quad (2)$$

where σ_0^2 is the absolute and σ_2^2 the relative part of the variance. The computed values for the above system ($\sigma_0 = 0.07$, $\sigma_2 = 0.04$) indicated that no weighting of the data was needed in the calculations (owing to the

relatively low value of the relative error; see ref. [15]). The data obtained at other pH values and/or with other substrates had similar error values. The identity of intercepts and the collinearity of pairs of straight lines were investigated by means of Student's *t* test [18]. The apparent K_m values for the tested substrates were determined from the initial-rate measurements at pH 7 and 25 °C by least-square fits using the v_0 -versus- $v_0/[\text{substrate}]$ linearization [19].

The stopped-flow experiments were carried out at 5 °C or 25 °C in an Aminco–Morrow apparatus equipped with a tungsten/iodine lamp and an electronic unit (DASAR); the data were processed by a computer. The absorption spectra were recorded with a Cary 118 spectrophotometer.

RESULTS AND DISCUSSION

Reduction of various arylnitroso substrates

Our results indicated that both aldehyde reductases 1 and 2 from pig livers were able to reduce several aromatic nitroso substances with NADPH as cofactor. This was evidenced by absorbance decreases at both 340 and 440 nm of the reaction mixtures containing these compounds upon the addition of enzyme. The nitroso substrates tested (except for nitrosobenzene) were reduced at higher maximal rates than was *p*-nitrobenzaldehyde, which is a good substrate for aldehyde reductase 1 [2] (see Table 1). The K_m values for these substrates are comparable with that for *p*-nitrobenzaldehyde [2] (except for *p*-nitroso-*N*-phenylaniline, which exhibits an essentially lower K_m value than the reference substrate). *p*-Nitroso-*N*-phenylaniline proved to be a very convenient substrate for aldehyde reductase 1. The analytical use of this substrate is not discussed in detail in the present paper, but its advantages are its good catalytical properties (see Table 1) and its high absorption coefficient at 440 nm; its only disadvantage is the non-negligible non-enzymic reduction in the presence of higher NADPH concentrations at pH values below 8. The values summarized in Table 1 are in accord with the reported positive influence of the substrate hydrophobicity on its kinetic behaviour with respect to

aldehyde reductase 1 [20, 21]. The most hydrophobic compound listed in Table 1 (i.e. *p*-nitroso-*N*-phenylaniline is the best arylnitroso substrate for aldehyde reductase 1, and *p*-nitroso-*N*-diethylaniline is better than *p*-nitroso-*N*-dimethylaniline. It may be noted that the aldehyde analogues of *p*-nitrosophenol and *p*-nitroso-*N*-dimethylaniline (*p*-hydroxybenzaldehyde and *p*-dimethylaminobenzaldehyde) are poor substrates for aldehyde reductase 1. This means that a suitable substituent of the arylnitroso compound is essential for the proper formation of the reaction product (see below), although it can be unfavourable for the reduction of the corresponding aldehyde. The unsubstituted nitrosobenzene is nearly unreactive with aldehyde reductases (cf. Table 1 and see below). Preliminary experiments with a crude preparation of aldehyde reductase 2 from pig liver showed that this enzyme has a lower K_m for *p*-nitroso-*N*-dimethylaniline than has aldehyde reductase 1. This is in accord with the fact that aldehyde reductase 2 exhibits lower K_m values with the usual substrates of aldehyde reductase [3].

The effect of pH on the relative reaction rates of *p*-nitroso-*N*-dimethylaniline, *p*-nitrosophenol and *p*-nitroso-*N*-phenylaniline is shown in Fig. 1. Aldehyde reductase 1 showed optimum activity with *p*-nitroso-*N*-dimethylaniline at pH 5.5–6.0 (similarly as with *p*-nitrobenzaldehyde and pyridine-3-aldehyde [2]) and a normal pH-activity profile. On the other hand the pH-activity profiles with *p*-nitrosophenol and *p*-nitroso-*N*-phenylaniline were more complex, and the reactivity of these substrates might also be influenced by their deprotonation. It is obvious that the reduction of *p*-nitrosophenol and *p*-nitroso-*N*-phenylaniline becomes more difficult in

Table 1. Apparent Michaelis constants and maximal reaction rates for arylnitroso substrates of aldehyde reductase 1 at pH 7

The conditions of measurements are given in the Materials and methods section. Relative values of $V_{\max}$ are percentages of that obtained with *p*-nitrobenzaldehyde. The reaction rates were measured at 340 nm.

Substrate	K_m (mM)	Relative $V_{\max}$ (%)
Nitrosobenzene	Negligible activity	
<i>p</i> -Nitroso- <i>N</i> -dimethylaniline	0.51	390
<i>p</i> -Nitroso- <i>N</i> -diethylaniline	0.28	480
<i>p</i> -Nitrosophenol	0.21	160
<i>p</i> -Nitroso- <i>N</i> -phenylaniline*	0.022	530
<i>p</i> -Nitrobenzaldehyde	0.25	100

* The reaction rates were corrected for the non-enzymic reduction of *p*-nitroso-*N*-phenylaniline observed before the addition of aldehyde reductase 1.

Fig. 1. Reaction of arylnitroso substrates of aldehyde reductase 1 as a function of pH

○, *p*-Nitroso-*N*-dimethylaniline (2 mM); □, *p*-nitrosophenol (1 mM); ▽, *p*-nitroso-*N*-phenylaniline (0.1 mM). Reaction rates were measured spectrophotometrically at 340 nm in the presence of 0.1 mM-NADPH at 25 °C; 0.1 M-sodium phosphate (pH 5–8), 0.1 M-sodium pyrophosphate (pH 8.5–9) and 0.1 M-NaOH/glycine (above pH 9) buffers were used. The maximal relative rate for each compound represents 100%. Corrections for non-enzymic reactions were carried out (for the data below pH 7 for *p*-nitroso-*N*-dimethylaniline and *p*-nitrosophenol and for those below pH 8 for *p*-nitroso-*N*-phenylaniline).

media more alkaline than the pK values for these compounds. The spectrophotometrically determined pK for *p*-nitrosophenol was approx. 6.3, and the pK for *p*-nitroso-*N*-phenylaniline determined by us was about 10.0 (the published value is 9.8 [8]). These pK values coincide well with the additional breaks in the pH-dependencies for activities with *p*-nitrosophenol and *p*-nitroso-*N*-phenylaniline as compared with that for *p*-nitroso-*N*-dimethylaniline (cf. Fig. 1 and see below).

The data presented here document that aldehyde reductases are the enzymes that can contribute to the metabolism of aryl nitroso compounds in mammalian cells in addition to the actions of alcohol dehydrogenase and microsomal enzymes [22]. This finding confirms the hypothesis on the role of aldehyde reductases that was presented in ref. [23].

Kinetics of *p*-nitroso-*N*-dimethylaniline reduction

We compared the steady-state kinetics of the reduction of *p*-nitroso-*N*-dimethylaniline with NADPH in the presence of aldehyde reductase 1 with that of the same system containing *p*-nitrobenzaldehyde as the substrate. The data were taken in accordance with Radika & Northrop [24], who proposed a simple method for the analysis of kinetic mechanisms of alternative substrates. Both NADPH and substrate concentrations were varied in these experiments, and the reaction rates were extrapolated to saturating concentrations of *p*-nitroso-*N*-dimethylaniline and *p*-nitrobenzaldehyde. The resulting double-reciprocal plots are shown in Fig. 2. The t test [18] showed that both intercepts and slopes of the straight lines in Fig. 2(a) are different (at the 95% confidence level), whereas those in Fig. 2(b) can be considered to be identical (at the same confidence level). This means that the simple ordered mechanism ought to be excluded at

pH 7. As the slope of the straight line for *p*-nitroso-*N*-dimethylaniline in Fig. 2(a) is larger than that for *p*-nitrobenzaldehyde, the prerequisites for the validity of the ordered mechanism seem to be violated for *p*-nitroso-*N*-dimethylaniline rather than for the aldehyde. Provided that the ordered mechanism is valid, then the slope ought to equal the reciprocal of the rate constant for the formation of the enzyme-NADPH complex, but in more complex cases it should increase, owing to the involvement of other rate constants [14, 24]. On the other hand, the results obtained at pH 8.5 might be compatible with the ordered reaction sequence (with NADPH binding before substrate), in view of the parallel straight lines in Fig. 2(b) [24]. As these straight lines nearly coincide (the difference of their intercepts is statistically insignificant), the Theorell-Chance approximation might be applicable in this case.

The complete kinetic data for changing concentrations of both NADPH and *p*-nitroso-*N*-dimethylaniline are shown in Fig. 3. These data rule out the improbable ping-pong mechanism at both pH values, since the straight lines obtained are not parallel [14]. The values of the kinetic coefficients (see the Materials and methods section) are summarized in Table 2. The ϕ_{12}/ϕ_2 ratio should equal the dissociation constant of the enzyme-NADPH complex if the basic kinetic mechanisms (such as ordered, preferred pathway or rapid-equilibrium random reaction sequences [14]) were involved. As the determined values of ϕ_{12}/ϕ_2 ($2.2 \pm 0.8 \mu\text{M}$ at pH 7 and $1.8 \pm 0.4 \mu\text{M}$ at pH 8.5) approach the published value of the equilibrium dissociation constant determined for aldehyde reductase from pig kidney at pH 7 ($1.3 \mu\text{M}$; cf. ref. [4]), some of these mechanisms might have to be taken into account. If the equilibrium random mechanism were valid the ϕ_{12}/ϕ_2 ratio should be equal to the dissociation

Fig. 2. Comparison of the steady-state kinetics for aldehyde reductase 1 with *p*-nitroso-*N*-dimethylaniline and *p*-nitrobenzaldehyde

Reaction rates were determined from A_{340} changes at pH 7 (a) and pH 8.5 (b) at 25 °C. The points represent the extrapolated values for saturating concentrations of *p*-nitroso-*N*-dimethylaniline (○) and *p*-nitrobenzaldehyde (●). The data for ten concentrations of substrates (in triplicate) were used for calculations of individual points. The t values for the slopes and intercepts in (a) were 2.03 and 3.01 respectively, and those for the slopes and intercepts in (b) were 0.26 and 0.46 respectively (the critical t value amounts to 1.94 at the 95% confidence level).

Fig. 3. Initial-rate measurements of the reduction of *p*-nitroso-*N*-dimethylaniline with NADPH catalysed by aldehyde reductase 1

Reaction rates were determined from A_{440} changes at 25 °C. (a) Primary plots obtained at pH 7; the NADPH concentrations were (for the straight lines downwards) 1, 2, 4, 10 and 40 μM . (b) Primary plots for the reactions at pH 8.5; the NADPH concentrations were (for the straight lines downwards) 0.5, 1, 2, 5 and 20 μM .

Table 2. Kinetic coefficients for the reduction of *p*-nitroso-*N*-dimethylaniline with NADPH and in the presence of NADP^+ and cyclohexanol

The coefficients are those of eqn. (1) for the data shown in Figs. 3 and 4; the conditions of measurements are described in the text. NADP^+ at 1 mM (saturating concentration) was used in the recyclization experiments (third line). The given errors of kinetic coefficients were greater than the differences in their values in repeated sets of experiments.

Conditions	ϕ_0 (s)	ϕ_1 ($\mu\text{M}\cdot\text{s}$)	ϕ_2 ($\mu\text{M}\cdot\text{s}$)	ϕ_{12} ($\mu\text{M}^2\cdot\text{s}$)
NADPH (pH 7)	0.024 ± 0.004	0.29 ± 0.03	12.9 ± 1.6	29 ± 7
NADPH (pH 8.5)	0.045 ± 0.005	0.26 ± 0.02	4.8 ± 0.3	8.6 ± 2.1
NADP^+ + cyclohexanol (pH 8.5)	0.41 ± 0.06	2500 ± 300	22.5 ± 1.4	95000 ± 20000

constant of the enzyme-*p*-nitroso-*N*-dimethylaniline complex, whereas ϕ_1/ϕ_0 and ϕ_2/ϕ_0 ratios should reflect the dissociation constants of the ternary enzyme-NADPH-*p*-nitroso-*N*-dimethylaniline complexes [14]. As $\phi_1/\phi_0 > \phi_{12}/\phi_2$ and $\phi_2/\phi_0 > \phi_{12}/\phi_1$ at pH 7 (see Table 1), the rapid-equilibrium random mechanism would imply a labilization of NADPH and *p*-nitroso-*N*-dimethylaniline binding in the ternary enzyme-NADPH-*p*-nitroso-*N*-dimethylaniline complex. Such a labilization is very improbable with NADP^+ - and NADP^+ -dependent dehydrogenases [14].

As the ordered mechanism seems to be invalid at pH 7 (see above), a more general non-equilibrium random mechanism (such as the preferred-pathway mechanism [14]) is probable for the *p*-nitroso-*N*-dimethylaniline reduction at pH 7. The occurrence of a significant inhibition at the excess of *p*-nitroso-*N*-dimethylaniline (above 1.5 mM), which was observed at pH 7, can also be reconciled with the preferred-pathway mechanism [14]. The inhibition of aldehyde reductase 1 by high substrate concentrations has been described in several cases, and the explanations of these phenomena have been similar

to that presented here (cf., e.g., ref. [6]). The values of the kinetic coefficients of aldehyde reductase 1 for the reaction with *p*-nitroso-*N*-dimethylaniline (Table 2) are of the same order of magnitude as the values found for other substrates of aldehyde reductase 1 from pig liver [2] and aldehyde reductase from pig kidney [4].

Our results indicate that aldehyde reductase 1 from pig liver probably exhibits a preferred-pathway mechanism towards *p*-nitroso-*N*-dimethylaniline at pH 7, whereas the ordered sequence (with the possibility of the Theorell-Chance approximation) seems to be valid at pH 8.5. This mechanism change might be attributed to a decreased tendency for the kinetically significant formation of the binary enzyme-substrate complexes at higher pH values and to the changes in the values of some rate constants. The changes in ϕ_0 and ϕ_2 (see Table 2) suggest that the rate of substrate conversion might be increased and that of NADP^+ splitting from the enzyme might be decreased at higher pH values. The latter process might become the rate-limiting step (as required by the Theorell-Chance approximation [14]) at pH 8.5.

Kinetics of the recyclization in the presence of *p*-nitrosodimethylaniline and cyclohexanol

The reduction of *p*-nitroso-*N*-dimethylaniline (and similar compounds) proceeds very quickly in the case of the alcohol dehydrogenase catalysis [7–9]. The nitroso compounds are unique in that the rate-limiting step for their chemical transformation by alcohol dehydrogenase appears to be their diffusion to the enzyme active sites [8]. The reduction of these substances catalysed by alcohol dehydrogenase can be carried out in a recycling manner in the presence of a nitroso substrate, NAD⁺ and a suitable alcohol [25–27]. The reaction rates of these recyclization reactions with alcohol dehydrogenase surpass essentially those of both direct *p*-nitroso-*N*-dimethylaniline reduction with NADH and alcohol oxidation with NAD⁺ [25–27]. We tried to find a suitable alcoholic substrate for such a recyclization of aldehyde reductase 1. This was rather difficult owing to the fact that alcohol oxidations catalysed with aldehyde reductase proceed relatively slowly [4]. We tested about ten possible alcoholic substrates of aldehyde reductase 1 with NADP⁺ as the coenzyme (at pH 7–10). Cyclohexanol and methylcyclohexanol proved to be the most convenient substrates. These substances were essentially better than glycerol, for which the kinetics of oxidation catalysed with kidney aldehyde reductase were analysed [4]. The kinetics of the coenzyme recycling reaction (at varied *p*-nitroso-*N*-dimethylaniline and cyclohexanol concentrations and saturating NADP⁺ concentrations at pH 8.5) was investigated (Fig. 4). The coefficients describing the kinetics of this reaction (Table 2) were computed. The reaction was described formally by the same equation

Fig. 4. Initial-rate measurements of the recyclization reaction catalysed by aldehyde reductase 1

The changes in A_{440} at 25 °C were evaluated. The reaction mixtures contained 1 mM-NADP⁺ at pH 8.5; the cyclohexanol concentrations were (for the straight lines downwards) 4, 8, 12 and 20 mM.

(eqn. 1) as that for a typical two-substrate reaction (see the Materials and methods section and refs. [26 and 27]).

The interpretation of the ϕ_0 coefficients is analogous for both direct and recyclization reactions [27] (cf. Table 2); this term corresponds to the reciprocal of the maximal reaction rate. As the value of ϕ_0 is essentially higher in the case of recyclization, this reaction cannot attain the rate of the direct reaction of *p*-nitroso-*N*-dimethylaniline with NADPH. This suggests that the rate-limiting steps in the direct and recyclization reactions are different. The value of the ϕ_2 coefficient (which should depend essentially on the rates of both *p*-nitroso-*N*-dimethylaniline reduction and cyclohexanol oxidation [27]) could approach the value of this term only if both substrates obey the Theorell–Chance mechanism [27]. In more-general mechanisms, ϕ_2 for the recyclization should be larger than ϕ_2 for the direct reaction [27]. As the tested system behaves in this way (cf. Table 2) and *p*-nitroso-*N*-dimethylaniline reduction approaches the Theorell–Chance mechanism at pH 8.5, the reaction of aldehyde reductase 1 with cyclohexanol cannot obey the Theorell–Chance mechanism (cf. also ref. [4]). The large values of ϕ_1 and ϕ_{12} for the recyclization confirm the fact that aldehyde reductase oxidizes alcohols at very low reaction rates (these coefficients should depend essentially on the reciprocal of the rate of the alcohol conversion [27]). However, the ϕ_1 term for cyclohexanol is essentially lower than the corresponding term for glycerol or pyridine-3-methanol [4].

The comparison of the maximal reaction rates of the described recyclization reaction with those of the direct reduction of *p*-nitroso-*N*-dimethylaniline in the presence of NADPH (0.1 mM) at pH 7–10 showed that the recyclization was 3–5 times slower than the direct reaction (cf. also the ϕ_0 values for pH 8.5 in Table 2). In the case of alcohol dehydrogenase the reaction rates of the recyclization surpassed essentially those of the direct reaction [26, 27] (owing to the fact that the slowest reaction step, i.e. the dissociation of NAD⁺ from the enzyme, was circumvented). In contrast, the alcohol oxidation seems to be the rate-limiting step in the recyclization reaction catalysed by aldehyde reductase 1 (see Table 2).

Although the cyclohexanol oxidation with NADP⁺ in the presence of aldehyde reductase 1 is slow, the splitting from the enzyme of the NADPH produced seems to be the rate-limiting step in the direct reaction of NADP⁺ and cyclohexanol. This is evidenced by the fact that the recyclization rates, determined from the A_{440} changes, were about one order of magnitude higher than the rates observed in the absence of *p*-nitroso-*N*-dimethylaniline at 340 nm in the presence of the same NADP⁺ and cyclohexanol concentrations (1 mM and 20 mM respectively) at pH 7–10. The approximate value of K_m was 0.1–0.15 mM, that for cyclohexanol amounted to about 3–5 mM, and substrate concentrations higher than 25 mM brought about a decrease in the reaction rate at pH 8.5. As these data on direct oxidation of cyclohexanol were rather scattered, no deeper analysis of this reaction was carried out. However, the fact that K_m for cyclohexanol is lower than the ϕ_1/ϕ_0 ratio for the recyclization (see Table 2) is in accord with the above-mentioned conclusions (cf. refs. [14] and [27]).

As the rate of the utilization of the enzyme–NADPH complex is at least 10-fold greater than the rate of its dissociation and the rate of the NADP⁺ → NADPH

conversion linked to the dehydrogenation of alcohol is small (see Table 2 and the discussion of the values of kinetic coefficients), the formation of the enzyme–NADPH complex should be negligible in the recyclization reaction. In accord with this assumption, no essential increase in A_{340} in the recyclization system was detected by the stopped-flow method. The more-sensitive fluorescence measurements could not be carried out for the demonstration of the NADPH production, since the nitroso compounds strongly quench the NADPH fluorescence.

Chemical mechanism of the *p*-nitrosodimethylaniline reduction

The formation of the quinonedi-iminium cation as the reaction product of the reduction of *p*-nitroso-*N*-dimethylaniline with NADH catalysed by alcohol dehydrogenase was proved by Koerber *et al.* [8] by several methods. We used two of the described methods in the case of *p*-nitroso-*N*-dimethylaniline reduction catalysed by aldehyde reductase (see Scheme 1). In stopped-flow experiments (40 μ M-*p*-nitroso-*N*-dimethylaniline and 0.1 mM-NADPH rapidly mixed with approx. 2 nM-aldehyde reductase at pH 8.5) the absorbance changes at 440 and 555 nm were registered. The value of A_{440} (corresponding to *p*-nitroso-*N*-dimethylaniline) decreased to zero within approx. 0.5 s under the given conditions, whereas a slower absorbance increase at 555 nm (maximal at about 0.7–1 s) was observed. The changes in A_{555} should reflect the formation of a relatively unstable *NN*-dimethyl-*p*-phenylenediamine radical cation [see reaction (b) in Scheme 1 and cf. ref. [8]]. This compound was formed from the primary reaction product (quinonedi-iminium cation) by the reaction with the excess of NADH in the presence of alcohol dehydrogenase [8]; its rapid formation by the reduction of the quinonedi-iminium cation with NADPH is still more probable owing to the more-negative redox potential of NADPH [28]. If lower NADPH concentrations (below 50 μ M) were used in these experiments the formation of the radical cation was essentially depressed (negligible absorbance increases at 555 nm were observed). As the concentration of this compound is maximal after the A_{440} value has fallen to zero, the *NN*-dimethyl-*p*-phenylenediamine radical cation cannot represent the primary product of the enzymic reaction. The same arguments were also used in

the analysis of the analogous reaction catalysed with alcohol dehydrogenase [8].

The formation of the quinonedi-iminium cation as the reaction product of the enzymic action of aldehyde reductase 1 on *p*-nitroso-*N*-dimethylaniline was confirmed by the addition of 1 mM-salicylamide (cf. refs. [8] and [29]) into the reaction mixture containing 0.1 mM-NADPH, 0.2 mM-*p*-nitroso-*N*-dimethylaniline and approx. 1 nM-aldehyde reductase 1 at pH 8.5. The increase in the absorbance at 680 nm corresponding to the indaniline dye formed from the quinonedi-iminium cation and salicylamide at the excess of NADPH [reaction (c) in Scheme 1] is in accord with the assumption that the quinonedi-iminium cation is the first (indirectly) detectable reaction product formed from *p*-nitroso-*N*-dimethylaniline in the presence of NADPH and aldehyde reductase 1 (cf. Scheme 1 and ref. [8]). The chemical mechanism of the *p*-nitroso-*N*-dimethylaniline reduction catalysed by aldehyde reductase 1 seems to be identical with that already proved for alcohol dehydrogenase [8].

The reactions of the other nitroso substrates (see Table 1) might proceed in a similar manner. The presence of a suitable substituent in the *para* position of the substrate molecule seems to be necessary for the stabilization of the products formed by the reduction and for the proper elimination of the OH^- ion from the reduced substrates (see Table 1). The effect of pH on the reduction rates of *p*-nitrosophenol and *p*-nitroso-*N*-phenylaniline can also be understood from this point of view (cf. Table 1 and Scheme 2). These compounds can transform into the corresponding anions at increasing pH values [8]. As these anions form the tautomeric (and unreactive) structures containing the $=\text{N}-\text{O}^-$ group [8] (Scheme 2) the reactions of these substrates are essentially slowed down at increasing pH values. In accord with this interpretation, the detailed analysis of the kinetics with *p*-nitrosophenol and *p*-nitroso-*N*-phenylaniline at different pH values showed that the decreases in the reaction rate observed at pH 6–8 and above pH 10 respectively were caused by increases in K_m values rather than by changes in maximal velocities. No acid–base changes come into account in the case of *p*-nitroso-*N*-dimethylaniline (and *p*-nitroso-*N*-diethylaniline) at pH 7–10 since the $\text{p}K$ values for these compounds are substantially lower ($\text{p}K$ for *p*-nitroso-*N*-dimethylaniline is about 4.1 [30]).

Scheme 1.

Scheme 2.

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