Modulation of the reactivity of the essential cysteine residue of betaine aldehyde dehydrogenase from *Pseudomonas aeruginosa*

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Betaine aldehyde dehydrogenase (BADH) catalyses the irreversible NAD(P)⁺-dependent oxidation of betaine aldehyde to glycine betaine. In the human opportunistic pathogen *Pseudomonas aeruginosa* this reaction is an obligatory step in the assimilation of carbon and nitrogen when bacteria are growing in choline or choline precursors. As with every aldehyde dehydrogenase studied so far, BADH possesses an essential cysteine residue involved in the formation of the intermediate thiohemiacetal with the aldehyde substrate. We report here that the chemical modification of this residue is conveniently measured by the loss in enzyme activity, which allowed us to explore its reactivity in a pH range around neutrality. The pH dependence of the observed second-order rate constant of BADH inactivation by methyl methanethiosulphonate (MMTS) suggests that at low pH values the essential cysteine residue exists as thiolate by the formation of an ion pair with a positively charged residue. The estimated macroscopic pK values are 8.6 and 4.0 for the free and ion-pair-forming thiolate respectively. The reactivity towards MMTS of both thiolate forms is notably lower than that of model compounds of similar pK, suggesting a considerable steric inhibition by the structure of the protein. Binding of the dinucleotides rapidly induced a significant and transitory increment of thiolate reactivity, followed by a relatively slow change to an almost unreactive form. Thus it seems that to gain protection against oxidation without compromising catalytic efficiency, BADH from *P. aeruginosa* has evolved a complex and previously undescribed mechanism, involving several conformational rearrangements of the active site, to suit the reactivity of the essential thiol to the availability of coenzyme and substrate.

Key words: chemical modification, NAD(P)H-induced conformational changes, thiolate pK, thiolate reactivity.

INTRODUCTION

The human pathogen *Pseudomonas aeruginosa* is able to grow on choline as well as choline precursors as the sole carbon, nitrogen and energy source [1]. One obligatory step in the catabolism of these compounds is the irreversible NAD(P)⁺-dependent oxidation of betaine aldehyde to glycine betaine, catalysed by betaine aldehyde dehydrogenase (BADH, EC 1.2.1.8). Glycine betaine is one of the most effective osmoprotectants in bacteria, algae, higher plants and animals [2,3]. *P. aeruginosa* is able to thrive under osmotic stress, particularly in the presence of the osmoprotectant glycine betaine or compounds which can produce glycine betaine, such as phosphatidylcholine, acetylcholine, phosphocholine or choline [4,5]. Because both osmotic stress [6] and these compounds [5,7–9] are present in the tissues infected by the bacteria, it is conceivable that BADH is a key enzyme in the establishment and growth of the pathogen. Given the high prevalence of antibiotic-resistant strains of *P. aeruginosa*, it is desirable to design new chemotherapeutic agents against this opportunistic pathogen. A rational approach should rely on the identification of aspects in the biochemistry of the bacteria that can be exploited. One such target might be the enzyme BADH.

Although the physicochemical and kinetic properties of BADH have been studied [10,11], little attention has been devoted to the study of the amino acid residues in the active centre of this enzyme and no data are available on its reaction mechanism. On the basis of its likely analogy with the mechanism of other aldehyde dehydrogenases [12–14] it can be assumed that the first step in the BADH-catalysed reaction is the formation of a thiohemiacetal between an essential cysteine residue and the substrate betaine aldehyde. However, this proposal has not been tested experimentally. The primary sequence of BADH from *P. aeruginosa* has already been published as a result of the *Pseudomonas* Genome Project [15]. The BADH subunit has 490 residues and only four cysteine residues. Alignment of the *Pseudomonas* BADH sequence with all other known aldehyde dehydrogenase sequences [16] indicates that Cys²⁸⁶ is the only conserved cysteine residue and it is probably the nucleophile involved in the thiohemiacetal formation. A study of the site-directed mutant Cys²⁸⁶ → Ala confirmed this prediction (R. Velasco-Garcia and R. A. Muñoz-Clares, unpublished work).

To understand the mechanistic aspects of the BADH reaction in *Pseudomonas*, we modified the enzyme chemically in an attempt to investigate the factors affecting the reactivity of the essential cysteine residue. The reactivity of a protein’s thiol group is determined by several factors: (1) the fraction of the thiol that is present as the thiolate form; (2) the intrinsic reactivity of the thiolate, which is dependent on the basicity of this group; and (3) the accessibility of the thiolate group to the solvent. We therefore determined the pK of Cys²⁸⁶ because this defines the extent of ionization and the intrinsic reactivity of its thiol group at any given pH value. Chemical modification with methyl methanethiosulphonate (MMTS), a small reagent that reacts rapidly and specifically with thiol groups and introduces a small group (-SCH₃) into the protein [17], has been used. In addition, by

Abbreviations used: BADH, betaine aldehyde dehydrogenase; Ches, 2-(N-cyclohexylamino)ethanesulphonic acid; DTT, dithiothreitol; MMTS, methyl methanethiosulphonate; Nbs₂, 5,5'-dithiobis-(2-nitrobenzolic acid).

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comparing the reactivity of the essential thiol towards MMPS with that expected for model alkyl thiols of similar pK values, the accessibility of this group to the solvent can be inferred.

We report here that the essential Cys residue in the BADH molecule exists mostly as a thiolate ion in the pH range 4.5–9.0 as a result of the formation of an ion pair with a positively charged residue. The low reactivity of the apoenzyme thiolate suggests that this group is not fully exposed to the solvent. However, at pH 8.0 and below, its reactivity increases significantly on binding of the oxidized or reduced coenzymes. This increase is transitory because the reactivity progressively decreases to a fixed low value, most probably as a consequence of a relatively slow ligand-induced conformational rearrangement of the active site. The possible implications of this new complex mechanism of modulation of thiolate reactivity are discussed.

MATERIALS AND METHODS

Chemicals and biochemicals

Betaine aldehyde chloride, glycine betaine (inner salt), NAD(P)+, NAD(P)H, 5,5′-dithiobis-(2-nitrobenzoic acid) (Nbs2), MMPS, dithiothreitol (DTT), Mes, 2-(N-cyclohexylamino)ethanesulphonic acid (Ches) and trehalose were obtained from Sigma (St Louis, MO, U.S.A.). EDTA was from Merck KGaA (Darmstadt, Germany). All other chemicals of analytical grade were from standard suppliers.

Enzyme purification and assay

BADH was purified to homogeneity from P. aeruginosa PAO1 strain by the rapid purification procedure reported previously [10]. The purity of the enzyme was assessed by SDS/PAGE as described previously [10]. The enzyme does not contain bound NAD(P)+ or NAD(P)H as judged by the A280/A260 of 2.0. The measurement of the dehydrogenase activity was performed spectrophotometrically by monitoring the increase in A340 (ε 6220 M⁻¹·cm⁻¹) in a 0.5 ml reaction mixture containing 1.0 mM betaine aldehyde and 0.5 mM NADP+ in a 50 mM potassium phosphate buffer, pH 8.0 (standard assay). A thermostatically controlled Philips PU 8710 spectrophotometer equipped with a kinetics software package was used for the assays, which were conducted at 30 °C. Assays were started by the addition of the enzyme. Steady-state rates were determined from the initial, linear portions of reaction progress curves. Each determination was performed at least in duplicate. Different enzyme preparations gave essentially the same results. One unit of activity is defined as the amount of enzyme catalysing the formation of 1 pmol of NADPH per min in our standard assay. The specific activity of the enzyme preparation used in this work, determined in the standard assay, was 80 units/mg of protein. Protein concentrations were determined by the Coomassie-G dye binding technique of Bradford [18], with BSA as a standard.

Reaction of BADH with MMPS

Before treatment of the enzyme with MMPS, the BADH preparations were gel-filtered twice by the method of Penefsky [19] to remove 2-mercaptoethanol. The gel-filtration buffer, buffer A, contained 50 mM potassium phosphate, pH 7.5, 1 mM EDTA, 20% (v/v) glycerol and 25 mM KCl; it was saturated with nitrogen just before use. MMPS and the enzyme ligands were dissolved in nitrogen-saturated water. BADH (final concentration 0.5 μM) was incubated with appropriate concentra-

Tions of MMPS in the absence or presence of ligands in buffer A at 30 °C. At appropriate intervals, aliquots of the reaction mixture were removed for the measurement of enzyme activity by the standard assay. Activity data were analysed by non-linear regression calculations with a commercial computing program formulated with the algorithm of Marquardt [20]. First-order analyses of time courses of inactivation were performed with:

$$E_t/E_a = e^{-kt}$$ (1)

for a monophasic inactivation or

$$E_t/E_a = E_1/E_a e^{-kt} + E_2/E_a e^{-kt}$$ (2)

for biphasic inactivation, where $E_t$ and $E_a$ are respectively the activity of the enzyme at times $t$ and 0; $k_1$, $k_2$ and $k_3$ are the observed pseudo-first-order rate constants and $E_1$ and $E_2$ are the amplitudes of each phase.

The order of the reaction, n, with respect to MMPS was determined by application of the equation described by Levy et al. [21]:

$$\ln k_{obs} = \ln k_{inact} + n \ln [\text{MMPS}]$$ (3)

where $k_{obs}$ is the observed pseudo-first-order rate constant of inactivation, as above, $k_{inact}$ is the second-order rate constant of inactivation, and n is the reaction order with respect to MMPS.

The inactivation of BADH by MMPS was reversed by the addition of excess DTT. Aliquots were assayed for activity at the indicated times. First-order analysis of the time courses of the reactivation reaction were performed with the following equation:

$$E_t/E_a = E_a/(E_a+(1-E_a/E_0))$$ (4)

where $E_t$, $E_a$ and $E_0$ are respectively the activity of the enzyme at times $t$ and zero and at equilibrium (time = ∞) and $k$ is the observed pseudo-first-order rate constant of reactivation.

The effect of dinucleotides on BADH inactivation by MMPS was investigated by preincubating the enzyme with the oxidized and reduced coenzyme for different times, as indicated in the Results section, before the addition of MMPS. Residual activity data obtained during the course of the inactivation reaction were fitted to eqn (2).

Thiol titrations with Nbs2

Thiol groups were determined on native and MMPS-modified BADH (0.9 μM) by means of Nbs2 titration [22], with a 200-fold excess of the reagent over the enzyme concentration. Incubations were performed with or without SDS (0.1%, w/v), at 30 °C in 50 mM phosphate buffer, pH 8.0, containing 1 mM EDTA. The number of modified cysteine residues was obtained from the time-dependent increase in A340 in a thermostatically controlled Philips PU 8710 spectrophotometer. The absorbance recorded was corrected for the blank (the reaction mixture minus the protein). The number of thiol groups was calculated from a molar absorption coefficient of 13600 M⁻¹·cm⁻¹ [22] for the Nbs2− chromophore liberated. The observed changes in absorbance, A, were fitted to a single-exponential equation:

$$A = A_0(1 - e^{-kt}) + c$$ (5)

where $A_0$ is the amplitude of the absorbance change, $k$ is the observed pseudo-first-order constant and $c$ is the absorbance at zero time. Excess thiol reagent and ligands were removed by passage over a Sephadex G-25 (Pharmacia, Uppsala, Sweden) column. The samples were then titrated with Nbs2 under either native or denaturing conditions, as described above.
the presence of these co-solutes. The enzyme stability was determined by incubating it in the same pH values above 7.5 in the absence of these co-solutes. The effectiveness of the co-solutes in preventing enzyme inactivation was derived for a model with a single ionizable group, which was tested by the existence of an ion pair, the data were first fitted to:

\[ k_{\text{inact}} = (k_B \times 10^{0\mathcal{K}_A-p\mathcal{H}} + k_C \times 10^{0\mathcal{K}_B-p\mathcal{H}}) \\
/ (1 + 10^{0\mathcal{K}_A-p\mathcal{H}} + 10^{0\mathcal{K}_B-p\mathcal{H}}) \]

Finally, the inactivation results were fitted with:

\[ k_{\text{inact}} = (k_B \times 10^{0\mathcal{K}_A-p\mathcal{H}} + k_C \times 10^{0\mathcal{K}_B-p\mathcal{H}}) \\
/ (1 + 10^{0\mathcal{K}_A-p\mathcal{H}} + 10^{0\mathcal{K}_B-p\mathcal{H}} + 10^{0\mathcal{K}_C-p\mathcal{H}}) \]

which was derived for the mechanism depicted in Scheme 2; the terms are as defined in Scheme 2.

**RESULTS AND DISCUSSION**

**Kinetics of the inactivation of BADH by MMTS**

Incubation of *P. aeruginosa* BADH with MMTS in buffer A at pH 7.5 and 30°C resulted in a time-dependent inactivation of the enzyme following pseudo-first-order kinetics (Figure 1A). In the absence of MMTS, under otherwise identical incubation conditions, virtually no changes in enzymic activity were observed during the experiment. The completely inactivated enzyme recovered 100% of its initial activity when treated with excess DTT (Figure 1A). The reactivation process also followed first-order kinetics.

To test for possible non-covalent binding of MMTS to the enzyme before inactivation, we determined the dependence of the inactivation rate constant on MMTS concentration. As shown in Figure 1(B), the observed pseudo-first-order rate constant for inactivation increased linearly with MMTS concentration. The absence of curvature suggests that, over the concentration range used, no significant E-MMTS complex accumulates during inactivation. Under these experimental conditions, a second-order rate constant of 1.4 \( \times 10^3 \) M\(^{-1}\) s\(^{-1}\) was obtained from the slope of the plot in Figure 1(B). The loss of catalytic activity was first-order with respect to MMTS (Figure 1C).

The inactivation of *P. aeruginosa* BADH by MMTS was

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**Scheme 1** Mechanism for the ionization of an ion-pair forming thiolate

\[ K_{A} \text{ and } K_{B} \text{ are the microscopic dissociation constants of the proton from the thiol group. } K_{A} \text{ and } K_{B} \text{ are the microscopic dissociation constants of the proton from the positively charged group. } K_{a} \text{ and } K_{b} \text{ are the macroscopic dissociation constants of the proton from the species } A \text{ and } B \text{ respectively. } k_{a} \text{ and } k_{b} \text{ are the second-order rate constants for the reaction of the thiolate in the species } B \text{ and } C \text{ with MMTS respectively.} \]

**Scheme 2** Proposed mechanism for the ionization of the essential thiolate of BADH from *P. aeruginosa*

\[ K_{A}, K_{B} \text{ and } K_{C} \text{ are the macroscopic dissociation constants of the species from } A, B \text{ and } C \text{ respectively. } k_{a}, k_{b} \text{ and } k_{c} \text{ are the second-order rate constants for the reaction of the thiolate in the species } B, C \text{ and } D \text{ with MMTS respectively.} \]
 monitored by assaying both the NADP⁺-dependent and NAD⁺-dependent activities under a variety of experimental conditions. There was no significant difference in the rate of loss of the two activities (results not shown).

**Thiol quantification of BADH**

Titration of pure BADH with Nbs₂ demonstrated the presence of two exposed thiol groups per monomer (Table 1). In the presence of 0.2% SDS four thiol groups were titrated, in full agreement with the primary structure of the enzyme [15]. Previous treatment of BADH with 25 μM MMTS for 20 min, which led to a total loss of enzyme activity, resulted in the modification of three thiol groups because no cysteine residues could be titrated with Nbs₂ in the absence of SDS and one was titrated in its presence. When the preincubation duration with MMTS was extended up to 40 min, no cysteine residue could be titrated by Nbs₂ in either the absence or presence of SDS, suggesting that the four cysteine residues were accessible to the thiosulphonate reagent.

The effects of saturating concentrations of the aldehyde substrate and dinucleotides on the number of accessible thiol groups was investigated by treating the enzyme with 25 μM MMTS for 20 min in the presence of saturating concentrations of NADP(H) and betaine aldehyde, alone or in combination. Under these conditions, most of the enzyme activity was protected against MMTS inactivation. After desalting to eliminate MMTS and ligands, Nbs₂ titration of the treated enzyme yielded only one reactive SH group, both in the absence of SDS and in its presence (Table 1). Because the protected enzyme retained most of its initial activity, we conclude that this group belongs to the essential cysteine residue, which was protected against MMTS modification by the active site ligands. This result indicates that the chemical modification of the essential cysteine residue is adequately measured by the loss of enzymic activity. In addition, this finding shows that the binding of substrate, either the aldehyde or the dinucleotides, exposes an additional thiol group. This is the same group that did not react with MMTS in the absence of ligands but under otherwise identical conditions. This effect is most probably due to a ligand-induced conformational change.

**pH dependence of the inactivation of BADH by MMTS**

The S-methylation of cysteine thiol groups with a reagent such as MMTS is well characterized; it has been shown that the thiolate anion is the reactive species [17]. Consequently, the measurement of the reaction rate as a function of pH can be used to determine the pKₐ values of the thiol group.

The reaction of MMTS with BADH was performed under pseudo-first-order kinetics in a cationic buffer in the presence of 600 mM trehalose or 20% (v/v) glycerol, as described in the Materials and methods section. As shown in Figure 2, the pH profile obtained in the presence of glycerol seemed shifted both to the right and downwards when compared with that obtained in the presence of trehalose, suggesting that glycerol causes a

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**Table 1 Thiol quantification of active and MMTS-treated BADH**

<table>
<thead>
<tr>
<th>Addition</th>
<th>Activity (% initial)</th>
<th>−SDS</th>
<th>+SDS</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100 ± 1</td>
<td>2.1</td>
<td>4.1</td>
</tr>
<tr>
<td>MMTS</td>
<td>0.0 ± 0.0</td>
<td>0.0</td>
<td>1.0</td>
</tr>
<tr>
<td>MMTS/DTT</td>
<td>100 ± 2</td>
<td>2.3</td>
<td>4.3</td>
</tr>
<tr>
<td>MMTS + BA</td>
<td>79 ± 1</td>
<td>0.95</td>
<td>0.9</td>
</tr>
<tr>
<td>MMTS + NADP⁺</td>
<td>76 ± 2</td>
<td>0.8</td>
<td>1.1</td>
</tr>
<tr>
<td>MMTS + NADP⁺ + BA</td>
<td>97 ± 3</td>
<td>1.1</td>
<td>0.0</td>
</tr>
<tr>
<td>MMTS + NADPH + BA</td>
<td>96 ± 1</td>
<td>0.8</td>
<td>0.2</td>
</tr>
</tbody>
</table>

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**Figure 1 Inactivation of *P. aeruginosa* BADH by MMTS**

(A) Time course of inactivation and reactivation by DTT. Enzyme (0.4 μM) was incubated with 5 μM MMTS at 30 °C in 50 mM potassium phosphate buffer, pH 7.5, containing 1 mM EDTA, 20% (v/v) glycerol and 25 mM KCl. At the times indicated, aliquots were withdrawn and assayed for remaining activity. The pseudo-first-order rate constant estimated from a fit of the data to eqn (1) was 0.49 ± 0.03 min⁻¹. After 30 min of reaction, 20 mM DTT was added to the inactivated enzyme and the recovery of activity was followed for an additional 90 min. The best fit of the reactivation data to eqn (4) gives an estimated reverse first-order rate constant (k⁻) of 0.042 ± 0.004 min⁻¹. The points are the experimental data and the lines are the best fit of these data. Results are means ± S.E.M. for three independent experiments. (B) Determination of the reaction order on MMTS from the data in (A) and assayed for remaining activity as a function of time. The observed pseudo-first-order rate constants for inactivation, k_obs, were estimated by fitting the experimental data to eqn (1). Results are means ± S.E.M. for three independent experiments. (C) Determination of the reaction order on MMTS from the data in (B), from eqn (3).
greater perturbation than trehalose in the pK of the thiolate group, diminishing its reactivity. However, because we could not determine the pK of this group in the absence of a co-solute, we cannot conclude whether these differences are due to solvent perturbation or to specific co-solute effects.

In both profiles the second-order rate constant for the inactivation of P. aeruginosa BADH by MMTS decreased as the pH of the incubation medium was lowered, reaching a plateau at low pH values. Assuming that a protonated thiol is not reactive towards MMTS [23], the theoretical line for the pH dependence of \( k_{\text{inact}} \) of a simple thiol should reach zero at low pH. For BADH, the pH profile of \( k_{\text{inact}} \) clearly deviates from that expected for a simple thiol. However, the experimental data can be fitted to eqn (6), corresponding to a mono-sigmoidal profile in which the protonated thiol group is still reactive towards MMTS (fit not shown). The estimated values for the thiol pK in the presence of trehalose or glycerol were 8.63 ± 0.05 and 9.04 ± 0.03 respectively; the limiting second-order rate constants at high and low pH values, \( k_H \) and \( k_L \), respectively, were 3.8 × 10^4 and 60 M\(^{-1}\) s\(^{-1}\) when trehalose was present, and 2.9 × 10^4 and 95 M\(^{-1}\) s\(^{-1}\) in the presence of glycerol. Because at low pH the thiol group is mostly protonated, whereas at high pH it is deprotonated, these results indicate that the protonated thiol is approx. 600–300-fold less reactive toward MMTS than the thiolate form [23]. Moreover, for a model compound such as 2-mercaptoethanol, it is known that the value of the second-order rate constant for reaction with MMTS at pH values below pH 5.65 is constant and less than \( 6 \times 10^{-4} \) M\(^{-1}\) s\(^{-1}\) [23]. The latter value is about five orders of magnitude lower than those found by us for BADH inactivation at low pH.

The most plausible reason for the existence of the plateau is therefore that the essential Cys remained ionized in the pH range explored and that the change in its reactivity was due to the titration of another group, as has been found for several other enzymes [24–29]. This could be explained if the essential Cys residue exists as an ion pair with a positively charged residue, as shown in Scheme 1. In this mechanism the macroscopic acid dissociation constants for the thiolate in the ion pair, \( K_A \), and the free thiolate, \( K_B \), are related to the microscopic acid dissociation constants by the following relationships:

\[
K_A = K_{11} + K_{12} \\
K_B = K_{12} \times K_{22}/(K_{12} + K_{22})
\]

Eqn (9) predicts that the microscopic pK of the thiolate in the ion pair, \( pK_{12} \), should be higher than \( pK_A \); eqn (10) predicts that the microscopic pK of the free thiolate is lower than \( pK_B \).

Because this is a plausible mechanism, we attempted to fit our data to eqn (7), which describes a reaction involving an ion-pair system. Given the impossibility of obtaining experimental data at low pH values because of enzyme instability, we could not obtain enough data to determine unequivocally the macroscopic pK and reactivity of the thiolate in the ion pair, \( pK_A \) and \( pK_B \) respectively, in Scheme 1. Moreover, there is a mutual dependence between parameters \( k_H \) and \( pK_{11} \) in eqn (7), so unless the value of \( pK_{11} \) is determined by other means the value of \( k_H \) cannot be known for certain. Given these uncertainties, we tentatively fitted the values of \( pK_A \), \( pK_{12} \) and \( k_H \) manually, considering that the value of \( pK_{11} \) should be slightly higher than that of \( pK_A \). Otherwise the pK for the positively charged group, \( pK_{12} \), would be unreasonably low, as deduced from eqn (9). Then we kept these parameters fixed to obtain, by non-linear regression, the macroscopic pK and the reactivity of the free thiolate, \( pK_B \) and \( k_L \) respectively in Scheme 1. The broken lines in Figure 2 show the fits when \( pK_{11} \) was set at 4.02, \( pK_A \) at 4.0 and \( k_H \) at 500 and 215 M\(^{-1}\) s\(^{-1}\) in the presence of trehalose and glycerol respectively. The estimated values for \( k_H \) and \( pK_B \) were (3.9 ± 0.14) × 10^4 M\(^{-1}\) s\(^{-1}\) and 8.66 ± 0.05 when trehalose was present, and (3.06 ± 0.15) × 10^4 M\(^{-1}\) s\(^{-1}\) and 9.07 ± 0.04 in the presence of glycerol. As can be seen in Figure 2 (broken lines), at low and high pH a relatively good fit was obtained with eqn (7) but several experimental data in the pH range 5.5–7.5 clearly deviated from the theoretical line resulting from this fit. The deviation was consistently present in both data sets, those obtained in the presence of trehalose or glycerol. These deviations of the experimental from the expected values might indicate that the protonation status of a third group was affecting the reactivity of the thiolate, as in the mechanism depicted in Scheme 2. In P. aeruginosa BADH, deprotonation of this enzyme group seems to restrict sterically the accessibility of the essential thiolate to the solvent, thus decreasing its reactivity, whereas deprotonation of the positively charged residue increases the reactivity of the essential thiol, by increasing its pK (which in turn increases its intrinsic reactivity) and/or its exposure to the solvent. Accordingly, our data were then best fitted to eqn (8), which was derived for the model in Scheme 2. We set \( pK_A \) and \( k_H \) to the values given above, manually fitted \( pK_B \) and \( k_L \) and obtained, by non-linear regression, the values of \( pK_B \) and \( k_L \). The solid lines in Figure 2 show the fit to eqn (8); the estimated values for the parameters are given in Table 2.

There have been numerous reports on the chemical modification of the essential thiol group of aldehyde dehydrogenases [30–35]. Moreover, the mechanism of the irreversible inhibition of aldehyde dehydrogenases by disulfiram, a drug widely used in aversion therapy for alcoholism, or its metabolites has been proved to involve the modification of this essential group [36–40]. However, there have been few studies on the factors modulating its reactivity. To the best of our knowledge these studies have been conducted only on phosphorylating [26,29,41,42] and non-phosphorylating [43] glyceraldehyde-3-phosphate dehydro-
genases, although possible mechanisms for the enhanced thiol reactivity of the cytoplasmic sheep liver aldehyde dehydrogenase have been discussed [44]. Chemical activation of the thiol of the essential Cys residue by ion-pair formation with a positively charged residue has been found in some cases [26,42]. Our results suggest that the macroscopic $pK$ of the essential thiol in *P. aeruginosa* BADH is also lowered by ion-pair formation. Although we could not precisely determine the extent of this decrease, owing to experimental difficulties arising from enzyme instability at low pH, we can conclude from our data that this $pK$ should be 4 or lower. We do not yet know the identity of the positively charged residue forming the ionic pair with Cys 286. On the basis of a three-dimensional model of *P. aeruginosa* BADH obtained from the crystal coordinates of cod liver BADH [45] (model not shown), the only possible candidates are Lys 286 and His 333, which in the model are respectively 10 and 13 Å from Cys 286 (1 Å = 0.1 nm). These residues seem too distant to be involved in charge–charge interactions with the essential cysteine residue but we cannot rule out its participation because these measurements were made on a model. Site-directed mutagenesis experiments are under way to define whether any of these residues are involved in the ion-pair formation.

It is known that the thiolate basicity, i.e. the thiol $pK$, affects its intrinsic reactivity, measured here as the rate constant for MMTS modification, $k_s$, in a way predicted by the Bronsted equation:

$$\log k_s = \log G + \beta pK \quad (11)$$

Considering the experimentally determined values for alkyl thiols of 3.54 for log $G$ and of 0.309 for $\beta$ [27], the reactivity of a simple alkyl thiolate of $pK$ 4.0 should be $5 \times 10^3$ M$^{-1} \cdot$s$^{-1}$, whereas that of a thiolate of $pK$ 8.66 or 9.07 should be $1.6 \times 10^6$ or $2.2 \times 10^6$ M$^{-1} \cdot$s$^{-1}$ respectively. Thus the reactivity of Cys 286 towards MMTS seems to be significantly lower than expected, about 120–270-fold when forming an ion pair and about 40–70-fold when it is a free thiolate. The reactivity of protein thiols depends not only on the group in the thiolate form or on the basicity of the thiolate but also on its accessibility, i.e. whether it is on the protein's surface or buried. It therefore seems that there is significant steric inhibition of the reaction of Cys 286 with MMTS that is partly related at high pH.

If the thiolate in species B and C were fully exposed to the solvent, the measured reactivity of these species would be related to the microscopic acid dissociation constants of the thiolate $K_{11}$ and $K_{22}$ by the relationship [28]:

$$k_B = k_s (10^{\beta(K_{11} - pK_{22})}) \quad (12)$$

However, eqn (12) cannot be applied to *P. aeruginosa* BADH owing to different steric effects of the protein environment on the thiolate reactivity at low and high pH values. It is therefore impossible to calculate the $pK$ for the free thiolate, $pK_{22}$, even if we assume that the $pK$ in the ion pair, $pK_{11}$, is that estimated by us.

**Effect of dinucleotides on the reactivity of the essential cysteine residue**

To determine the $pK$ of the essential thiol group in the binary complex enzyme–dinucleotide, we investigated the pH dependence (in the pH range 5.5–9.0) of the pseudo-first-order rate constant of inactivation at saturating concentrations (6 mM) of NADP$^+$, which is about 100-fold the corresponding $K_s$ for NADP$^+$ over the whole pH range studied. Incubations were performed in Mes/Hepes/Ches buffer plus 600 mM trehalose, as described in the Materials and methods section. We found that incubation of the enzyme with MMTS in the presence of NADP$^+$ resulted in a biphasic loss of catalytic activity: a first phase with a high thiolate reactivity was followed by a second phase with a much lower reactivity (results not shown). The apparent pseudo-first-order rate constant for inactivation and the amplitude of the two phases were therefore estimated from a fit of the data to a double-exponential equation [eqn (2)]. Second-order rate constants for inactivation were then determined by dividing those estimates by the MMTS concentration. Figure 3 shows the pH profile for the reactivity of the essential cysteine residue in the enzyme–NADP$^+$ complex, as measured by the second-order rate constant of the first inactivation phase. This profile shows clearly that the thiolate in the holoenzyme is still chemically activated by forming an ion pair with a positively charged residue, which on deprotonation increases the $pK$ of the thiol and consequently its reactivity. In addition, in the presence of saturating concentrations of NADP$^+$ the reactivity of the thiolate increases significantly at pH values of 8.0 and lower, in comparison with that obtained in the absence of the dinucleotide. The binding of NADP$^+$ largely eliminates the effect of the residue named YH in Scheme 2 on the reactivity of the essential thiolate, so the
inactivation data obtained for the holoenzyme can be fitted to eqn (7) by following the same procedure as above, i.e. by manually fitting the reactivity and the pK for the thiolate in the ion pair, $K_\text{p}$, and $pK_\text{Cys}$, and estimating by non-linear regression the values of the reactivity and the pK of the free thiolate, which are given in Table 2 as $k_{\text{fast}}$ and $pK_\text{Cys}$, respectively. The results of this fit are shown in Table 2. The estimated macroscopic pK for the free thiolate is 1.2 pH units lower than that estimated in the absence of NADP$. Because this value should be higher than any of the microscopic pK values of the two groups involved in ion pairing, it sets a higher limit for the free thiolate pK. The second-order rate constant for modification of the thiolate in the ion pair is 10-fold higher in the holoenzyme than in the apoenzyme, whereas that for the free thiolate is 2.7-fold smaller. The latter decrease in thiolate intrinsic reactivity is consistent with that expected if the pK of the group is 1.2 pH units lower, in line with eqn (11). Qualitatively, the same activation effects were observed with NAD+ and NAD(P)H (results not shown); they are therefore not attributable to chemical activation by the positive charge of NADP$^+$. These findings provide evidence for a conformational activation of the essential Cys$^{286}$ after dinucleotide binding, suggesting that at pH values below 8.0 this binding triggers a rearrangement of the active site, leading to a more ‘open’ conformation. The changes in thiolate reactivity were fully reversed when the nucleotides were removed from the enzyme preparation by desalting (results not shown).

It is interesting that as the pH was increased from 5.5 to 9.0 the ratio between the second-order rate constant for inactivation in the presence of NADP$, k_{\text{fast}}$, to that in its absence, $k$, progressively diminished until it reached approx. 0.7 at pH 9.0. The pH profile of this ratio, shown in the inset of Figure 3, suggests that the protonation of a group of pK 7.16$\pm$0.07 is involved in the rapid change in Cys$^{286}$ reactivity promoted by dinucleotide binding. It seems that the rapid dinucleotide-induced rearrangement of the active site, which result in a more ‘open’ conformation, requires that a certain group of the enzyme be protonated.

### Table 3 Effect of dinucleotides on the reactivity of the essential thiolate and kinetics of the slow dinucleotide-induced conformational change

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>$k_{\text{fast}}$ (M$^{-1} \cdot$ s$^{-1}$)</th>
<th>$k_{\text{slow}}$ (M$^{-1} \cdot$ s$^{-1}$)</th>
<th>$k_{\text{reactivity}}$ (s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAD+</td>
<td>(4.3$\pm$0.9) $\times 10^2$</td>
<td>147$\pm$30</td>
<td>84$\pm$12</td>
</tr>
<tr>
<td>NADP$^+$</td>
<td>(5.0$\pm$1.5) $\times 10^2$</td>
<td>123$\pm$43</td>
<td>158$\pm$44</td>
</tr>
<tr>
<td>NADH</td>
<td>(5.0$\pm$0.1) $\times 10^2$</td>
<td>127$\pm$33</td>
<td>85$\pm$32</td>
</tr>
<tr>
<td>NADPH</td>
<td>(7.3$\pm$1.1) $\times 10^2$</td>
<td>260$\pm$20</td>
<td>183$\pm$20</td>
</tr>
</tbody>
</table>

### Scheme 3 Proposed mechanism for the changes in the essential thiolate reactivity elicited by binding of the dinucleotides to BADH from P. aeruginosa

$E^*$ and $E^{\text{NAD(P)H}}$ are different holoenzyme conformations with a high- and low-reactive essential thiolate respectively.

Several reasons could account for the existence of the two distinguishable inactivation rates in the whole pH range studied when the dinucleotide-saturated BADH is treated with MMTS. The enzyme preparation is homogeneous, so the presence of isoenzymes cannot be the cause of this anomalous behaviour. Because P. aeruginosa BADH is dimeric, negative co-operativity between the two active sites in the reaction with MMTS could account for a biphasic inactivation process, i.e. modification of the first active site by MMTS could lead to a decreased reactivity of the second active site in the same BADH molecule. However, this possibility seems unlikely because such co-operativity was not observed in the absence of the dinucleotide. It is also possible that binding of NAD(P)$^+$ or NAD(P)H triggers an additional, and relatively slow, conformational change that eventually makes the thiolate less accessible to the solvent. To test the latter possibility, reaction of the enzyme with MMTS was initiated after different preincubation periods with NAD(P)$^+$ or NAD(P)H. Figure 4 depicts the time course of inactivation by MMTS at pH 8.0, with and without preincubation of the enzyme with NADH. Similar results were obtained with the other three dinucleotides (results not shown). We found that for each dinucleotide the whole set of residual activity data could be fitted to eqn (2), yielding the values of second-order rate constants for inactivation given in Table 3. As the preincubation time increased, the amplitude of the first rapid inactivation phase decreased, but the rate constants for the two phases remained unchanged. The minimal mechanism that accounts for these results is that in Scheme 3, in which the holoenzyme, after binding the dinucleotide, undergoes at least two conformational changes, the first leading to a form, $E^*$, with a very reactive

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**Figure 4** Effect of NADH on the inactivation of BADH by MMTS

BADH (0.5 μM) was inactivated with 5 μM MMTS in the absence (■) or presence of 6 mM NADH, which had been added to the enzyme 1 (●), 15 (○), 30 (○), 60 (●) and 120 (○) min before treatment with MMTS. The buffer used was 30 mM Mes/Hepes/Ches, pH 8.0, containing 1 mM EDTA, 25 mM KCl and 600 mM trehalose. Results are means±S.E.M. for two independent experiments. The data obtained in the absence of dinucleotide were fitted to eqn (1) and those obtained in the presence of dinucleotide were fitted to eqn (2). Inset: a plot of the amplitude of the first inactivation phase against time.
the holoenzyme might be deleterious because it increases the risk of oxidation, BADH from _P. aeruginosa_ has evolved a complex mechanism, involving several conformational rearrangements of the active site, to suit the reactivity of the essential thiol to the availability of dinucleotide and substrate.

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