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 p*K* 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 p*K*, 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 p*K*, 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, phosphorylcholine 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 Cys^{286} is the only conserved cysteine residue and it is probably the nucleophile involved in the thiohemiacetal formation. A study of the site-directed mutant $Cys^{286} \rightarrow Ala$ confirmed this prediction (R. Velasco-García 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 p*K* of Cys^{286} 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 ($-SCH_3$) 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; Nbs_2, 5,5′-dithiobis-(2-nitrobenzoic acid).

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comparing the observed reactivity of the essential thiol towards MMTS 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) (Nbs_2), MMTS, dithiothreitol (DTT), Mes, Hepes, 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 A_{280}/A_{260} of 2.0. The measurement of the dehydrogenase activity was performed spectrophotometrically by monitoring the increase in A_{340} (ϵ 6220 $M^{-1} \cdot cm^{-1}$) 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 μmol 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 MMTS

Before treatment of the enzyme with MMTS, 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. MMTS and the enzyme ligands were dissolved in nitrogen-saturated water. BADH (final concentration 0.5 μM) was incubated with appropriate concentrations of MMTS 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:

$$E_t/E_0 = e^{-kt} \quad (1)$$

for a monophasic inactivation or

$$E_t/E_0 = E_1/E_0 e^{-k_1 t} + E_2/E_0 e^{-k_2 t} \quad (2)$$

for biphasic inactivation, where E_t and E_0 are respectively the activity of the enzyme at times t and 0; k, k_1 and k_2 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 MMTS was determined by application of the equation described by Levy et al. [21]:

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

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 MMTS.

The inactivation of BADH by MMTS 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:

$$E_t/E_0 = E_\infty/E_0 + (1 - E_\infty/E_0)e^{-kt} \quad (4)$$

where E_t, E_0 and E_∞ 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.

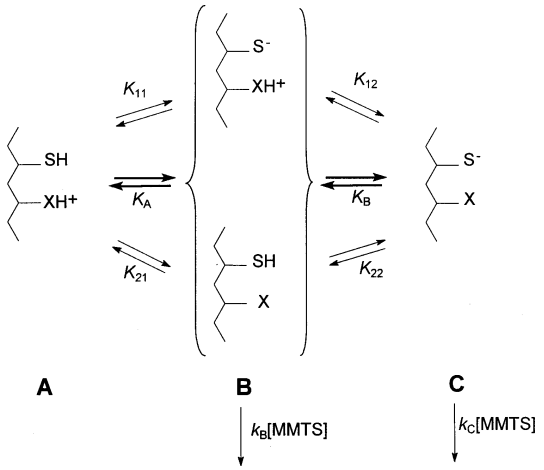
The effect of dinucleotides on BADH inactivation by MMTS was investigated by preincubating the enzyme with the oxidized and reduced coenzyme for different times, as indicated in the Results section, before the addition of MMTS. Residual activity data obtained during the course of the inactivation reaction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

Thiol titrations with Nbs_2

Thiol groups were determined on native and MMTS-modified BADH (0.9 μM) by means of Nbs_2 titration [22], with a 200-fold excess of the reagent over the enzyme concentration. Incubations were performed with or without SDS (0.2 %, 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 A_{412} 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 of a molar absorption coefficient of 13600 $M^{-1} \cdot cm^{-1}$ [22] for the Nbs^{2-} chromophore liberated. The observed changes in absorbance, A, were fitted to a single-exponential equation:

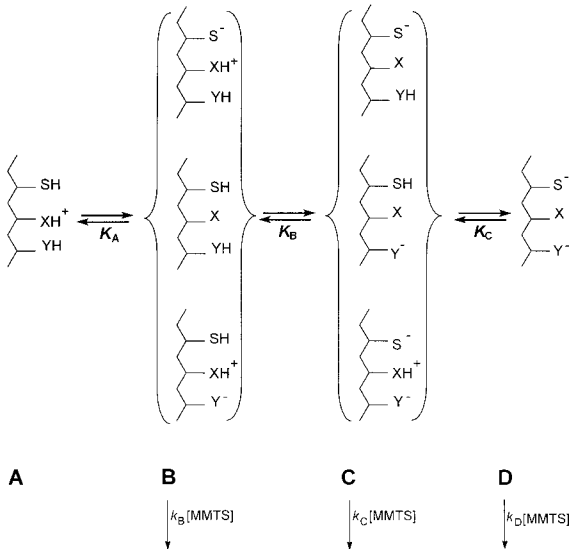
$$A = A_1(1 - e^{-kt}) + c \quad (5)$$

where A_1 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 Nbs_2 under either native or denaturing conditions, as described above.



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

K_{12} and K_{22} are the microscopic dissociation constants of the proton from the thiol group. K_{21} and K_{12} are the microscopic dissociation constants of the proton from the positively charged group. K_A and K_B are the macroscopic dissociation constants of the proton from the species A and B respectively. k_B and k_C are the second-order rate constants for the reaction of the thiolate in the species B and C with MMTS respectively.



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

K_A, K_B and K_C are the macroscopic dissociation constants of the species from A, B and C respectively. k_B, k_C and k_D are the second-order rate constants for the reaction of the thiolate in the species B, C and D with MMTS respectively.

pH studies

The pH dependence of the inactivation rate was obtained by incubating the enzyme (0.5 μM, final concentration) with 5 μM MMTS in a Mes/Hepes/Ches buffer (each at 30 mM), containing 1 mM EDTA and 25 mM KCl together with 600 mM trehalose or 20 % (v/v) glycerol, adjusted with KOH to the indicated pH values. We attempted to perform the pH studies in the absence of co-solutes, but *P. aeruginosa* BADH is rapidly inactivated at pH values above 7.5 in the absence of these co-solutes. The enzyme stability was determined by incubating it in the same buffer at 30 °C and at the pH of interest and taking aliquots for assay at pH 8.0 under standard conditions. The aliquots were taken at different intervals, which were as long, at least, as the incubation duration with the modifying reagent. The order of effectiveness of the co-solutes in preventing enzyme inactivation at high pH was 600 mM trehalose > 20 % (v/v) glycerol > 20 % (w/v) sucrose > 10 % (w/v) poly(ethylene glycol). We therefore used trehalose or glycerol as the stabilizing co-solute. The pH range in which the enzyme was stable for the incubation durations and at the temperature employed in these experiments was 4.5–9.5 in the presence of trehalose or 5.5–9.0 in the presence of glycerol. The rate constant of inactivation, k_{obs}, was determined over these pH ranges and the second-order rate constants, k_{inact}, were calculated by dividing k_{obs} by the concentration of MMTS. Results obtained for the pH dependence of k_{inact} were fitted to:

$$k_{inact} = (k_H + k_L \times 10^{pK-pH})/(1 + 10^{pK-pH}) \quad (6)$$

which was derived for a model with a single ionizable group, where k_H and k_L represent the second-order rate constants for the plateau at high and low pH values respectively. For testing the existence of an ion pair, the data were first fitted to:

$$k_{inact} = (k_B \times 10^{pK_A - pK_{11}} + k_C \times 10^{pH-pK_B}) / (1 + 10^{pK_A-pH} + 10^{pH-pK_B}) \quad (7)$$

where k_B and k_C are the second-order rate constants for the modification of the thiolate in the species B and C respectively (Scheme 1), pK_A and pK_B are the macroscopic pK values of the thiolate in species B and C respectively, and pK_{11} is the microscopic pK of the thiol group in the ion pair (see Scheme 1). Finally, the inactivation results were fitted with:

$$k_{inact} = (k_B \times 10^{pK_A-pH} + k_C + k_D \times 10^{pH-pK_B}) / (1 + 10^{pK_B+pK_A-2pH} + 10^{pK_B-pH} + 10^{pH-pK_C}) \quad (8)$$

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 1.4×10^3 $M^{-1} \cdot s^{-1}$ 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

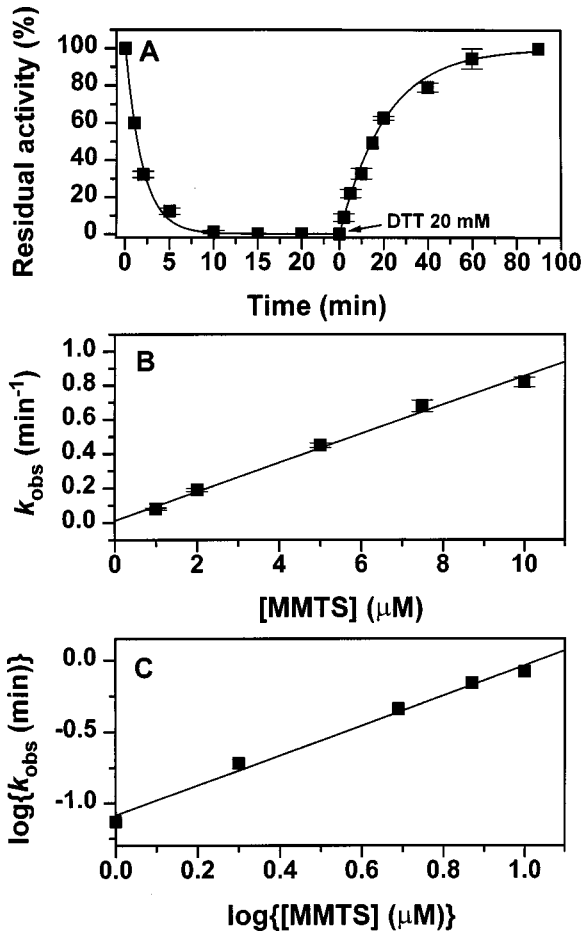


Figure 1 Inactivation of *P. aeruginosa* BADH by MMTS

(**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_{-1}) 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 $\pm$ S.E.M. for three independent experiments. (**B**) Dependence of the rate constant for inactivation on MMTS concentration. BADH was incubated with the indicated concentrations of MMTS as 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 $\pm$ S.E.M. for three independent experiments. (**C**) Determination of the reaction order on MMTS from the data in (**B**), from eqn (3).

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 Nbs_2 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 Nbs_2 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 Nbs_2 in either the absence or presence of SDS, suggesting that the four cysteine residues were accessible to the thiosulphonate reagent.

Table 1 Thiol quantification of active and MMTS-treated BADH

Native and MMTS-treated BADH (0.9 μM) were incubated with Nbs_2 in 50 mM phosphate buffer, pH 8.0, containing 25 mM KCl, 1 mM EDTA and 20% (v/v) glycerol; thiol quantification was performed as described in the Materials and methods section. For MMTS modification, the enzyme was preincubated with 25 μM MMTS for 20 min in the absence and the presence of the indicated ligands. Then the mixture was gel-filtered twice to remove MMTS and ligands and used immediately for thiol quantification. The changes in A_{412} were fitted to eqn (5). Where noted, 20 mM betaine aldehyde (BA), 5 mM $NADP^+$ and 1.8 mM NADPH were present during the MMTS inactivation but were absent during thiol quantification. Results are means $\pm$ S.E.M. for two independent experiments.

		No. of thiol groups per monomer	
Addition	Activity (% initial)	−SDS	+SDS
None	100 ± 1	2.1 ± 0.3	4.1 ± 0.3
MMTS	0.0 ± 00	0.0 ± 0.0	1.0 ± 0.0
MMTS/DTT	100 ± 2	2.3 ± 0.0	4.3 ± 0.1
MMTS + BA	79 ± 1	0.95 ± 0.1	0.9 ± 0.0
MMTS + $NADP^+$	76 ± 2	0.8 ± 0.0	1.1 ± 0.1
MMTS + $NADP^+$ + BA	97 ± 3	1.1 ± 0.1	0.8 ± 0.1
MMTS + NADPH + BA	96 ± 1	0.8 ± 0.2	0.9 ± 0.1

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, Nbs_2 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 pK 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 seems shifted both to the right and downwards when compared with that obtained in the presence of trehalose, suggesting that glycerol causes a

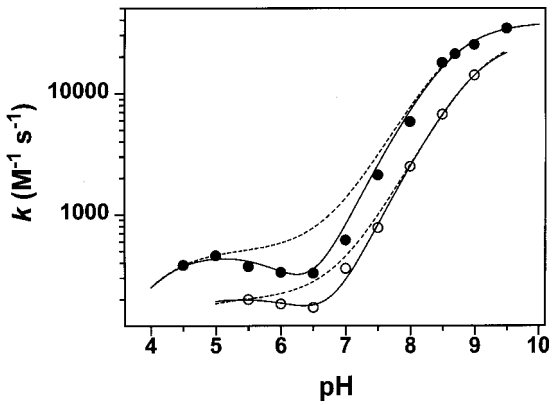


Figure 2 pH dependence of the second-order rate constant for the inactivation of *P. aeruginosa* BADH by MMTS

BADH (0.5 μM) was incubated with 5 μM MMTS at the indicated pH values. The buffer used was 30 mM Mes/Hepes/Ches containing 1 mM EDTA and 25 mM KCl together with 20% (v/v) glycerol (○) or 600 mM trehalose (●). Results are means from two independent experiments; S.E.M. values (less than 10% of means) are omitted, given the semilogarithmic nature of the plot. The solid lines were obtained from a non-linear least-squares fit of the data to eqn (8). The broken lines are the best non-linear fit to eqn (7).

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_{inact} of a simple thiol should reach zero at low pH. For BADH, the pH profile of k_{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 $M^{-1} \cdot s^{-1}$ when trehalose was present, and 2.9×10^4 and 95 $M^{-1} \cdot s^{-1}$ 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. It is therefore highly unlikely that this represents a reaction of the protonated thiol because it has been shown that this form is approximately nine or ten orders of magnitude less reactive with 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 6×10^{-4} $M^{-1} \cdot s^{-1}$ [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 a 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:

$$K_A = K_{11} + K_{21} \quad (9)$$

$$K_B = K_{12} \times K_{22}/(K_{12} + K_{22}) \quad (10)$$

Eqn (9) predicts that the microscopic pK of the thiolate in the ion pair, pK_{11}, 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 k_B respectively, in Scheme 1. Moreover, there is a mutual dependence between parameters k_B and pK_{11} in eqn (7), so unless the value of pK_{11} is determined by other means the value of k_B cannot be known for certain. Given these uncertainties, we tentatively fitted the values of pK_A, pK_{11} and k_B 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_{21}, 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_C 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_B at 500 and 215 $M^{-1} \cdot s^{-1}$, in the presence of trehalose and glycerol respectively. The estimated values for k_B and pK_B were $(3.9 \pm 0.14) \times 10^4$ $M^{-1} \cdot s^{-1}$ and 8.66 ± 0.05 when trehalose was present, and $(3.06 \pm 0.15) \times 10^4$ $M^{-1} \cdot s^{-1}$ 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_B to the values given above, manually fitted pK_B and k_C and obtained, by non-linear regression, the values of pK_C and k_D. 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-

Table 2 Second-order rate constants for the inactivation by MMTS and macroscopic p*K* values of the essential thiolate

Results shown are parameters (means ± S.E.M.) estimated from a fit of the inactivation data shown in Figure 2 (apoenzyme) to eqn (8) and those shown in Figure 3 (holoenzyme) to eqn (7). Parameters fitted manually are those without S.E.M.

		Rate constants ($M^{-1} \cdot s^{-1}$)			Macroscopic p*K* values		
Enzyme form	Co-solute	k_B	k_C	k_D	pK_A	pK_B	pK_C
Apoenzyme	Trehalose	500	5	$(3.83 \pm 0.13) \times 10^4$	4	6.2	8.63 ± 0.04
	Glycerol	215	48	$(2.86 \pm 0.03) \times 10^4$	4	6.6	9.01 ± 0.01
Holoenzyme	Trehalose	5000	–	$(1.40 \pm 0.05) \times 10^4$	4	–	7.44 ± 0.10

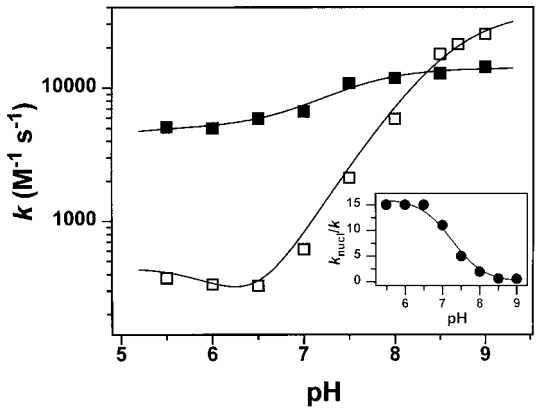


Figure 3 pH dependence of the second-order rate constant of inactivation of BADH by MMTS at saturating concentrations of $NADP^+$

BADH (0.5 μM) was incubated with 5 μM MMTS in the presence of 6 mM $NADP^+$ (added to the apoenzyme 2 min before treatment with MMTS) (■) at the indicated pH values. Results are means from two independent experiments. S.E.M. values (less than 15% of means) are omitted, given the semilogarithmic nature of the plot. The line is the best fit of the data to eqn (7). Data from Figure 2, obtained in the absence of $NADP^+$ but under otherwise identical experimental conditions, are included for comparison (□). Inset: pH profile of the ratio of the second-order rate constant observed in the presence of $NADP^+$, k_{nucl}, to the second-order rate constant observed in its absence, k. The line in the inset is the best fit of the points to the equation for a single ionizable group.

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 p*K* 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 p*K* 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^{96} and His^{333}, which in the model are respectively 10 and 13 Å from Cys^{286} (1Å ≡ 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 p*K*, affects its intrinsic reactivity, measured here as the rate constant for MMTS modification, k_s, in a way predicted by the Brønsted 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 β [27], the reactivity of a simple alkyl thiolate of p*K* 4.0 should be $5.9 \times 10^4\ M^{-1} \cdot s^{-1}$, whereas that of a thiolate of p*K* 8.66 or 9.07 should be 1.6×10^6 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 fraction of the group in the thiolate form or on the basicity of the thiolate but also on its accessibility, i.e. whether 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 relieved 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]:

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

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 p*K* for the free thiolate, pK_{22}, even if we assume that the p*K* 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 p*K* 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_d 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 p*K* 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

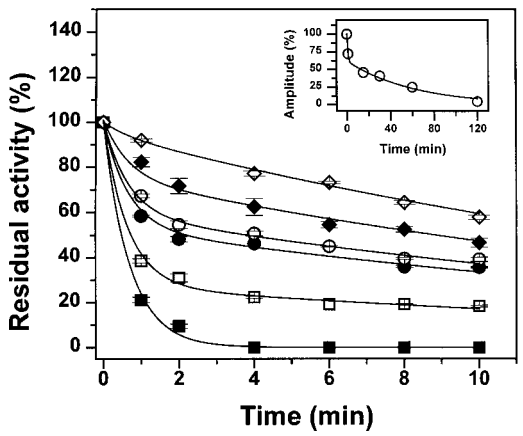


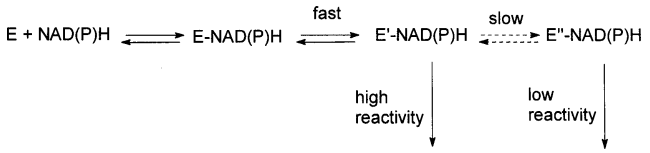
Figure 4 Effect of NADH on the inactivation of BADH by MMTS

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.

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

BADH (0.5 μM) was preincubated with 6 mM dinucleotides for different durations and then treated with 5 μM MMTS under the conditions given in the legend to Figure 4. The thiolate reactivity of the two holoenzyme forms E′ and E′′ is expressed as the second-order rate constant for the first and second phase of inactivation respectively. Results shown are parameters estimated from a fit of the data (means ± S.E.M. for two independent experiments) to eqn (2). The kinetics of the slow dinucleotide-induced conformational change is given as the first-order rate constant of the two phases, k_{obs1} and k_{obs2}, for the change in the relative concentration of E′ (see the text for details). Eqn (2) was used to fit these data.

	Thiolate reactivity		Conformational change	
Nucleotide	k_{inact1} ($M^{-1} \cdot s^{-1}$)	k_{inact2} ($M^{-1} \cdot s^{-1}$)	k_{obs1} (s^{-1})	k_{obs2} (s^{-1})
NAD^+	$(4.3 \pm 0.9) \times 10^3$	147 ± 30	84 ± 12	1.9 ± 0.2
$NADP^+$	$(5.0 \pm 1.5) \times 10^3$	123 ± 43	158 ± 44	1.3 ± 0.0
NADH	$(5.0 \pm 0.1) \times 10^3$	127 ± 33	85 ± 32	0.9 ± 0.2
NADPH	$(7.3 \pm 1.1) \times 10^3$	260 ± 20	183 ± 20	1.3 ± 0.2



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′′ are different holoenzyme conformations with a high- and low-reactive essential thiolate respectively.

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_B and pK_A, 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_D and pK_C respectively. The results of this fit are given 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 later 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_{nucl}, 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 ± 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.

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

thiolate followed by another conformational change resulting in a form, E'', with a thiolate at least 40-fold less reactive than the former. The relative concentrations of both forms are given by the amplitudes of the two phases. The inset to Figure 4 shows the time course of the conformational change elicited by NADH, as determined by the changes in the amplitude of the first inactivation phase. The best fit of these data was obtained by using a double-exponential equation, considering that at zero time of preincubation 100% of the holoenzyme is already in the conformation E'. This indicates that the conformational change leading to the initial increase in thiolate reactivity is so fast that it cannot be resolved by standard procedures and that the conformational change leading to a very unreactive thiolate takes place in at least two steps, with the first-order rate constants given in Table 3. The kinetics of the conformational changes triggered by the four dinucleotides tested are qualitatively identical and quantitatively very similar, although the conformational changes induced by NADP(H) seem to be faster than those induced by NAD(H).

Taken together, our results indicate that the binding of dinucleotides has two effects on the reactivity of thiolate towards MMTS: (1) a transitory increase in its reactivity at pH values below 8.5, resulting from a very rapid conformational change, and (2) a subsequent large decrease in reactivity as a result of a slow conformational change. Marchal and Branlant [43] have reported dinucleotide-induced changes in the reactivity of the essential thiolate of the non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase from *Streptococcus mutans*. However, their findings differ significantly from ours in that they observed a rapid initial decrease in the reactivity of the thiolate on binding of the dinucleotide, followed by a slow increase in its reactivity.

Both dinucleotide-induced conformational changes are reversed on removal of the dinucleotide. The slow conformational change is presumably rapidly reversed after binding of the aldehyde to the holoenzyme, because the enzyme activity at saturating substrate concentration was not affected by preincubation of the enzyme with the dinucleotide (results not shown). However, we were unable to measure the reactivity of the essential thiol in the presence of both substrates, given the high level of protection against MMTS inactivation afforded by the aldehyde.

We can speculate about the possible implications that our findings on the modulation of the reactivity of the essential thiolate at pH values around neutrality have for BADH activity. The active-site conformation of the apoenzyme results in a low reactivity of the essential thiol, even though most of it is as thiolate. This 'close' conformation most probably protects the apoenzyme from inactivation by oxidation. Accordingly, we have found that *P. aeruginosa* BADH can remain active for a long period after removal of the reductant used to protect it against oxidation by ambient O_2. The binding of the dinucleotide rapidly increases Cys^{286} reactivity, most probably because of an increase in the accessibility of its thiol group to the solvent. This in turn results in an enhanced rate of thiohemiacetal formation with the aldehyde substrate. Thus the rapid dinucleotide-induced conformational change probably produces a holoenzyme with a higher affinity than the apoenzyme for betaine aldehyde. The kinetic mechanism of *P. aeruginosa* BADH is consistent with this proposal, because the dinucleotide is the preferred first substrate [11]. In contrast, the high reactivity of the essential thiol group in the holoenzyme might be deleterious because it increases the risk of oxidation of this group. Therefore the slow dinucleotide-induced change, resulting in an enzyme conformation with an almost unreactive essential thiol, would give substantial protection against holoenzyme inactivation. This protective effect might be relevant if the aldehyde concentrations are below those required for enzyme saturation. Thus it seems that, to gain protection against thiolate oxidation without compromising catalytic efficiency, 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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