Borate inhibits activation of inactive dinitrogenase reductase from *Rhodospirillum rubrum*

Paul W. LUDDEN

Department of Biochemistry, University of California, Riverside, CA 92521, U.S.A.

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Borate and aminophenylboronic acid were tested as inhibitors of activation of inactive dinitrogenase reductase from *Rhodospirillum rubrum*. Inhibition was specific for activation because activity of the active form of the enzyme was not inhibited. Inhibition showed the pH-dependence expected if borate inhibits by binding to *cis*-hydroxy groups of the modifying group found on the inactive enzyme.

The nitrogenase reductase (Fe protein) from *Rhodospirillum rubrum* exists in active and inactive forms (Ludden & Burris, 1976, 1978; Nordlund *et al.*, 1977; Carithers *et al.*, 1979). When cells are grown with glutamate as the N source, the inactive form of the enzyme is obtained, even though active nitrogenase is detected in the whole cells (Ludden & Burris, 1976). The enzyme purified from glutamate-grown cells is inactive by virtue of a covalent modification (Ludden & Burris, 1979) that consists of pentose, phosphate and an adenine-like molecule (Ludden & Burris, 1978). Removal of the adenine-like molecule results in activation of the enzyme. Removal is effected by an activating enzyme (AE) that can be isolated from the chromatophore fraction of the broken cells. ATP is required for removal of the adenine-like compound from native reduced protein, but ATP is not required for removal of the adenine-like compound from oxidized dinitrogenase reductase. The modification found on Rr_{2i} does not appear to be AMP or ADP-ribose.

Borate is known to bind to *cis*-hydroxy groups of carbohydrates (Edwards *et al.*, 1955; Lorand & Edwards, 1959) and thus is inhibitory to some reactions in which a ribose-containing cofactor is used by an enzyme. Borate is normally avoided as a

buffer for NAD-dependent dehydrogenase for this reason. In the present study, borate and aminophenylboronic acid were tested as inhibitors of activation of the Fe protein of nitrogenase from *R. rubrum* because inactive protein has a pentose attached. It was thought that binding of borate to *cis*-hydroxy groups might interfere with activation of the enzyme.

Materials and methods

Preparations of proteins

Nitrogenase components were purified as previously described (Ludden & Burris, 1978). Rr_{2i} was purified from cells which had been grown with glutamate as the N source. Rr_{2a} was purified by the same method from cells grown with limiting NH₄⁺ as the N source (Carithers *et al.*, 1979). Alternatively Rr_{2a} was prepared from Rr_{2i} *in vitro* by treatment with AE and MgATP²⁻ plus Mn²⁺ under anaerobic conditions. The active form of Rr₂ was then separated from AE by column chromatography on DEAE-cellulose (Ludden & Burris, 1976). Rr₁ used in the present experiments was purified by elution from DEAE-cellulose by an NaCl gradient followed by precipitation with poly(ethylene glycol) *M*_{r,av.} 4000). Rr₁ at this stage of purification is not homogeneous on SDS/polyacrylamide gels, but it is highly active and free of Rr₂ and AE activities. AE used in assays was prepared by elution from buffer-washed chromatophores (membrane fragments) with 0.5M-NaCl and precipitated by 30% poly(ethylene glycol). This AE is slightly contaminated by Rr₁ but not by Rr₂. AE used in the preparation of Rr_{2a} from Rr_{2i} *in vitro* was further purified by chromatography on DEAE-cellulose before use.

Abbreviations used: Rr_{2i}, inactive form of dinitrogen reductase (the Fe protein); Rr_{2a}, the active form; AE, enzyme required for activation of Rr₂; Rr₁, dinitrogenase (the MoFe protein) from *Rhodospirillum rubrum* (these abbreviations were agreed to by a number of groups working on nitrogen fixation by photosynthetic bacteria at an informal meeting at the Fourth International Symposium on Nitrogen Fixation held in Canberra, Australia, December 1980); SDS, sodium dodecyl sulphate; Mes, 4-morpholine-ethanesulphonic acid; Mops, 4-morpholine-propanesulphonic acid.

Enzyme assays

Nitrogenase activity was assayed by acetylene reduction (Koch & Evans, 1966). Assays for $Rr2_a$ contained 5 mM-ATP, 25 mM-phosphocreatine, 50 μ g of creatine phosphokinase, 10 mM-sodium dithionite, 10 mM- $MgSO_4$ and enzymes as noted. Assays for activation of $Rr2_i$ included the above components, except that the $MgSO_4$ concentration was 25 mM and 0.5 mM- Mn^{2+} was added. In assays to determine the effect of borate concentration on activity, the buffer was 50 mM-Tris/HCl, pH 8.0. In assays to determine the effect of pH on the extent of borate inhibition, a mixture of Mes, Mops and Tris buffers, each at 50 mM, was used. Borate solutions were prepared from boric acid and adjusted to the pH of the assay with NaOH.

Results and discussion

Fig. 1 shows the effect of borate concentration on acetylene-reduction activity of a mixture of $Rr1$, $Rr2_i$ and AE in the presence of reductant and ATP. In a 15 min incubation, 50% inhibition of acetylene reduction was achieved at approx. 4 mM-borate at pH 8.0, and 90% inhibition was seen with 20 mM-borate. At higher pH values, even stronger inhibition was seen (up to 98%). Similar results were obtained when acetylene reduction was measured at 5 min; thus borate inhibits activation throughout the time course of activation.

Fig. 1. Effect of borate concentration on the activation of inactive dinitrogenase reductase by activating enzyme. Assay conditions are described in the text. Each incubation mixture contained 105 μ g of $Rr2_i$ and 45 μ g of $Rr1$; 100% activity equals 123 nmol of acetylene reduced in 15 min.

If borate were effecting activation by binding to the modifying group on $Rr2_i$, it would be expected that higher pH, which strengthens the *cis*-diol-borate complex, would enhance inhibition. Fig. 2 shows that higher pH does enhance inhibition of activation (as measured by coupling activation of $Rr2_i$ to the acetylene assay). At pH 6.5, the presence of 20 mM-borate in the assay mixture results in only 40% inhibition, whereas at pH 8.5, 95% inhibition is seen.

From the experiments described above, it is not clear whether borate inhibits activation of $Rr2_i$, the activity of $Rr2_a$ or some other component of the acetylene assay mixture. The effect of borate on the activity of active $Rr2$ is shown in Fig. 2. Borate (20 mM) inhibits the activity of $Rr2_a$ only 12% and no effect of pH on the extent of inhibition is seen. Neither $Rr2$ isolated in the active form from NH_4^+ -limited cells nor $Rr2$, which was activated *in vitro* before use, was inhibited strongly by borate. The only assay components that differ between the assay for active $Rr2$ and the assay for activation of inactive $Rr2$ are: (1) activating factor, (2) the form

Fig. 2. Effect of pH on borate inhibition of active and inactive forms of dinitrogenase reductase. Assay conditions are described in the text. Incubation mixtures contained 105 μ g of active or inactive enzyme and 45 μ g of $Rr1$. Maximal activity of $Rr2_a$ was 234 nmol of acetylene reduced in 5 min. ●, $Rr2_a$ in assay; ○, $Rr2_i$ in assay.

of Rr2 initially present in the assay and (3) the presence of 0.5 mM- Mn^{2+} in the assay for activation. Increasing the concentration of Mn^{2+} did not overcome the inhibition, and therefore the inhibition is not due to some interaction between Mn^{2+} and borate. The experiments described above have not ruled out the possibility that borate inhibits by binding to AE itself, but the more likely possibility is that borate binds to the modifying group found on inactive Rr2.

Aminophenylboronic acid also showed pH-dependent inhibition that was specific for activation. This compound is commonly used to affix borate to insoluble supports and can be readily attached to commercially available activated agarose (for example, Bio-Rad Affigel 10).

Arsenate and phosphate were also tested as inhibitors of activation. Arsenate (20 mM) showed 35% inhibition at pH 8.0 and 14% inhibition at pH 7.0. Phosphate at 20 mM showed 14% inhibition at pH 8.0 and 33% inhibition at pH 7.0. Neither compound was specific for activation in its inhibition, and the inhibition was not as strong as that seen with borate.

Activation of reduced native Rr2 requires the presence of ATP, and thus it is possible that it might exert its effect by binding to the ribose of ATP rather than the pentose of the modifying group. The effect of borate on the Walker & Mortenson (1973) assay for ATP binding to iron protein was tested. Only slight inhibition was seen, indicating that borate does not inhibit by binding to the ribose of ATP.

Borate was not effective in protecting whole cells of *R. rubrum* from inhibition of nitrogenase activity by NH_4^+ .

Borate affinity columns have been used to purify carbohydrate-modified peptides (Annamalai *et al.*, 1979; Van Ness *et al.*, 1978). Borate affinity columns may be useful in the purification of a tryptic fragment of Rr2₁, which contains the covalently bound modifying group.

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