Vol. 107, No. 1, 1982 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 16, 1982 Pages 12-18

NITRATE REDUCTASE FROM PENICILLIUM CHRYSOGENUM: KINETIC MECHANISM AT SUB-OPTIMUM pH

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Received May 4, 1982

The steady-state kinetics of the NADPH + FAD-dependent reduction of nitrate by nitrate reductase from Penicillium chrysogenum was studied at pH 6.18. At this sub-optimum pH, V_{max} was about 83 units x mg protein^{-1} compared with 225 units x mg protein^{-1} at pH 7.20. All initial velocity reciprocal plot patterns at pH 6.18 as well as the $NADP^+$/nitrate product inhibition pattern were intersecting. In contrast, the NADP(H)/nitrate plots at pH 7.20 were parallel (Renosto, F. et al. J. Biol. Chem. 256, 8616, 1981). A major effect of lowering the assay pH was to change the K_m for FAD from 0.17 μM at pH 7.20 to 4 μM at pH 6.18. The results suggest that nitrate reductase has a steady-state random kinetic mechanism in which k_{cat} in the forward direction at pH 7.20 (ca. 375 sec^{-1}) is greater than k_{off} for the dissociation of one or more substrates. Several observations suggest that k_{off} for FAD is extremely small at pH 7.20.

INTRODUCTION

Nitrate reductase (E.C 1.6.6.1-3) catalyzes the reduced pyridine nucleotide + flavin-dependent reduction of inorganic nitrate to nitrite. The assimilatory enzyme from Chlorella and higher plants prefers NADH, while the fungal enzyme prefers NADPH. The purified fungal enzyme also requires added FAD for activity, while the green plant enzymes are maximally active in the absence of added flavin coenzyme. (The plant enzymes contain tightly-bound FAD). During the past few years, studies on the kinetics of nitrate reductase from several sources have been published. However, there is still some question as to the kinetic mechanism. The enzymes from Ankistrodesmus braunii (a green alga) (1), corn and squash (2), spinach (3), and Penicillium chrysogenum (4) yield parallel NAD(P)H/nitrate initial velocity reciprocal plots which, together with the expected product inhibition patterns, suggest a non-classical, two-site, hybrid ping pong-rapid equilibrium random mechanism (5) with irreversible steps somewhere in the overall catalytic cycle (4), or

0006-291X/82/130012-07$01.00/0

an iso ping-pong mechanism (1,3). On the other hand, the enzymes from Chlorella vulgaris (6) and Aspergillus nidulans (7) yield intersecting NAD(P)H/nitrate plots, which, together with the expected product inhibition patterns (8) support a classical rapid equilibrium random mechanism.

There are two obvious explanations for the different kinetic patterns: (a) The enzymes from different sources have intrinsically different mechanisms (i.e., the relative magnitudes of the various rate constants are significantly different for different nitrate reductases). (b) Native nitrate reductase from all sources has the same kinetic mechanism, but a structural alteration occurring during purification or storage causes the ratios of certain rate constants to change. If, for example, the mechanism is steady-state random with the k_{off} for two of the substrates equal to about 1/3 k_{cat}, certain initial velocity plots would appear to be parallel and yield distorted values for some kinetic constants (9). A small structural change in the enzyme (e.g. proteolytic clipping) occuring in some purification procedures might decrease k_{cat}/k_{off} just enough to change the patterns from parallel to intersecting. The resulting initial velocity and product inhibition patterns would then be consistent with a rapid equilibrium random mechanism (and the mechanism would be interpreted as such) even though the interconversion of the central complexes (or release of the first product) was still not rate-limiting. The experiments described in this paper were designed to check this possibility. But rather than attempt to duplicate the purification and storage procedures used by others, or to deliberately induce limited proteolysis, we examined the kinetics of the reaction at a pH where k_{cat} was significantly reduced. Our hope was that the sub-optimum pH would not decrease the k_{off} values for substrate dissociation by exactly the same factor. Other methods for probing the basis of the parallel plots, such as studying the reverse reaction or using alternative substrates in the forward reaction (9) could not be used because (a) the reverse reaction does not proceed to a measurable extent, (b) NADH does not serve as an effective alternative reducing agent (4), (c) chlorate is almost as effective an oxidant as nitrate (4), while bromate is a better substrate than nitrate (unpublished results).

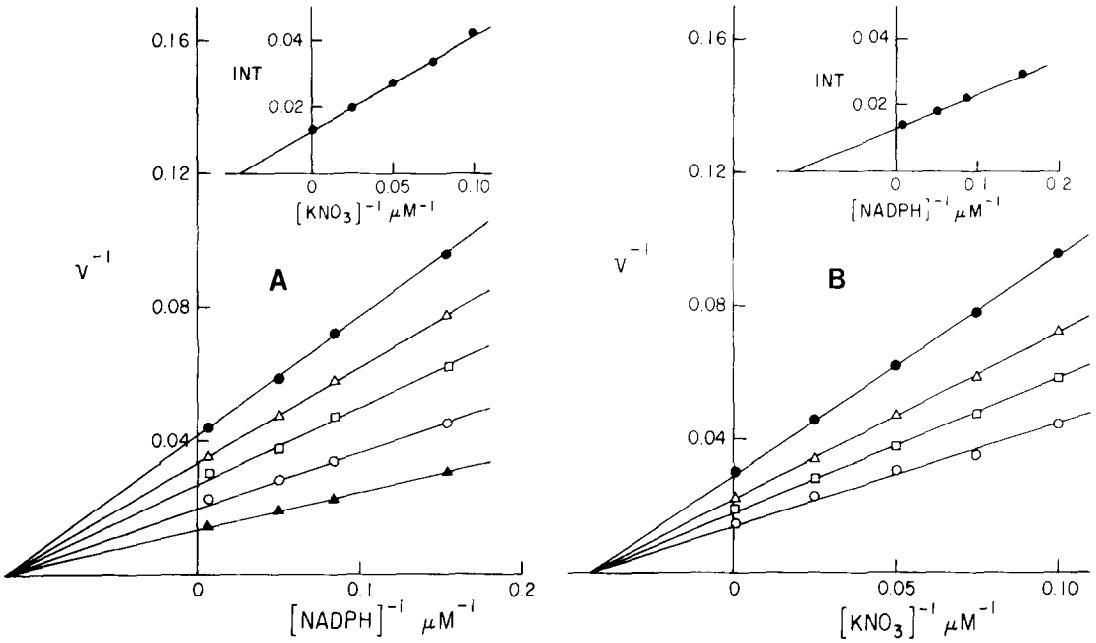

Figure 1. Initial velocity reciprocal plots showing the interaction of NADPH and nitrate at pH 6.18. The concentration of FAD was maintained constant at 22 μM. A. The fixed NADPH concentrations were 6.6 μM, 12 μM, 20 μM, 160 μM. B. The fixed KNO_3 concentrations were 10 μM, 13.3 μM, 20 μM, 40 μM, 10^4 μM. In all assays, the reaction was started by adding nitrate.

MATERIALS AND METHODS

Nitrate reductase was purified from induced mycelium of Penicillium chrysogenum as previously described (4) except that the Bio-Gel agarose A-0.5m column was replaced with a Sephacryl S-300 column (90 x 2.3 cm). Enzyme activity was measured as the decrease in NADPH absorbance at 340 nm as described previously (4). All experiments reported in this paper were carried out in 45 mM potassium phosphate buffer, pH 6.18 containing 0.5 mM Na_2EDTA. The enzyme used for the experiments had a specific activity of 225 units x mg protein^{-1} at pH 7.20. Velocities are reported in terms of μmoles x min^{-1} x mg protein^{-1} and are corrected for the slow non-enzymatic oxidation of NADPH at pH 6.18. Rates were not corrected for the low enzyme-catalyzed, nitrate-independent oxidation of NADPH because preliminary experiments using an oxygen electrode showed that this background (essentially, FAD reductase activity followed by spontaneous reoxidation of the $FADH_2$) was suppressed in the presence of nitrate. Generally, each assay mixture contained 0.02-0.04 μg enzyme per ml. NADPH utilization at the lowest NADPH concentrations used did not exceed 10% over the assay period.

RESULTS

Figures 1-3 show the initial velocity reciprocal plot patterns at pH 6.18. For each family of plots the third substrate was held constant at a concentration greater than five times its K_m. At constant FAD concentrations $<K_m$, the slopes of some of the 1/v versus $1/[NO_3^-]$ plots appeared to increase with NADPH concentration and several lines of the family of plots intersected to the right of the 1/v-axis (data not shown). This unusual

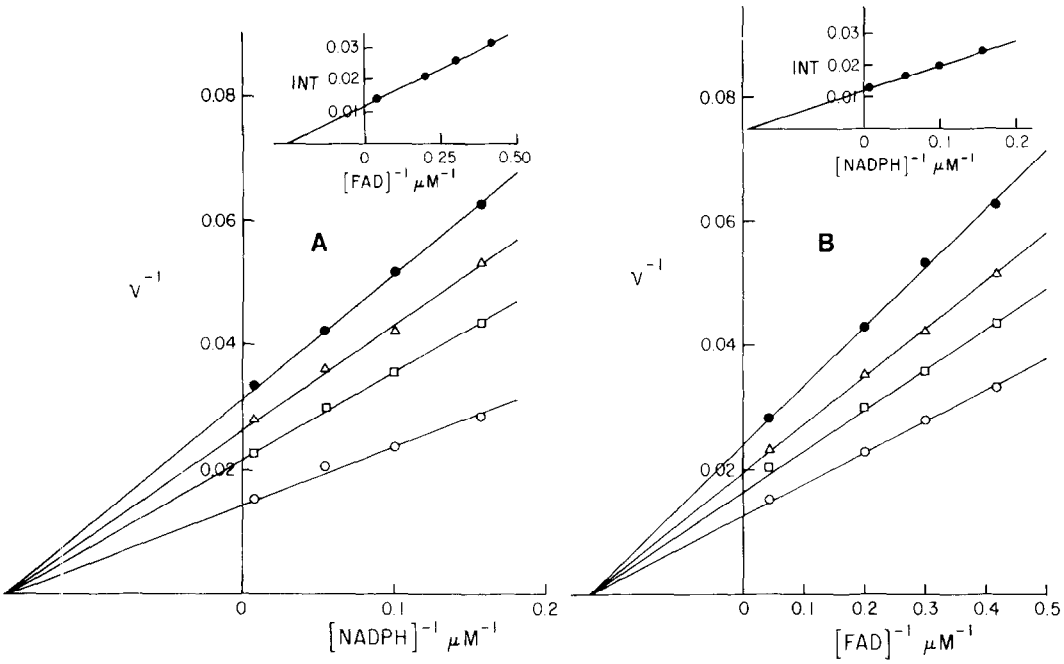

Figure 2. Initial velocity reciprocal plots showing the interaction of NADPH and FAD at pH 6.18. The concentration of nitrate was maintained constant at 10 mM. A. The fixed NADPH concentrations were 6.4 μM, 10 μM, 18.5 μM, 140 μM. B. The fixed FAD concentrations were 2.4 μM, 3.3 μM, 5 μM, 24 μM.

pattern is consistent with a steady-state random mechanism in which k_{off} for two substrates are each <1/3 k_{cat} (9). In other experiments (data not shown) nitrite was found to be competitive with nitrate and uncompetitive with respect to NADPH (the same patterns observed at pH 7.20). $NADP^+$ was competitive with NADPH and noncompetitive with respect to nitrate. Table 1 summarizes the kinetic constants and reciprocal plot patterns at pH 6.18 and pH 7.20.

DISCUSSION

At pH 7.20, P. chrysogenum nitrate reductase has a specific activity of 225 units x mg protein^{-1}, corresponding to an active site turnover number of 375 sec^{-1}. At pH 6.18, V_{max} is 83 units x mg protein^{-1}, corresponding to an active site turnover number of 138 sec^{-1}. The 2.7-fold decrease in k_{cat} was accompanied by a 4-fold decrease in the K_m for nitrate, a small change in the K_m for NADPH, and a 24-fold increase in the K_m for FAD. The NADPH/nitrate reciprocal plot patterns, which were parallel at pH 7.20 in the presence of saturating or unsaturating FAD (4) became intersecting at pH 6.18 and [FAD]

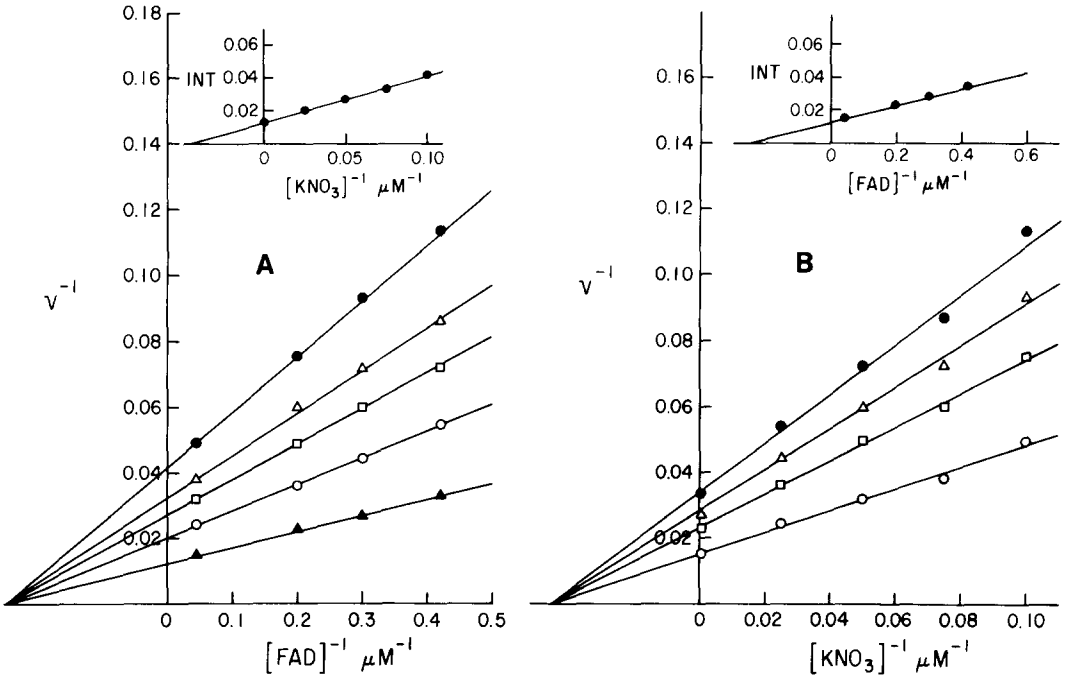

Figure 3. Initial velocity reciprocal plots showing the interaction of FAD and nitrate at pH 6.18. The concentration of NADPH was maintained constant at 170 μM. A. The fixed FAD concentrations were 2.4 μM, 3.3 μM, 5 μM, 22 μM. B. The fixed KNO_3 concentrations were 10 μM, 13.3 μM, 20.0 μM, 40 μM, 10^4 μM.

>5 K_m. The simplest explanation for the results is that the mechanism is predominantly steady-state random and the parallel NADPH/nitrate plots observed at pH 7.20 are a result of unusually small k_{off}/k_{cat} ratios (9). The change in the character of the NADPH/nitrate plots from parallel to intersecting as the pH is lowered may be partly a consequence of increasing k_{off} for FAD (as reflected in the 24-fold increase in the K_m for FAD).

Several observations are consistent with an extremely small k_{off} for FAD at pH 7.20: (a) The $k_{cat}/K_{m_{FAD}}$ ratio at pH 7.20 is 2 x 10^9 M^{-1} x sec^{-1}, which exceeds the second-order rate constant for the diffusion-limited encounter of a substrate and an enzyme (10). This suggests that k_{off} for FAD is so low that the coenzyme does not dissociate during each catalytic cycle. (b) FAD alone is ineffective in eluting nitrate reductase from an FAD affinity column at pH 7.20 (4). (High salt concentration together with FAD must be used.) It seems more reasonable that the extremely high affinity of the enzyme for the immobilized FAD reflects a very small k_{off} rather than a very high

TABLE 1
Effect of pH on the Kinetic Constants and Initial Velocity Reciprocal Plot Patterns of Nitrate Reductase from Penicillium chrysogenum

Constant or Pattern	pH 6.18	pH 7.20
V_{max}	83 units x mg^{-1}	225 units x mg^{-1}
K_m of nitrate	23 μM	91 μM
K_m of FAD	4 μM	0.17 μM
K_m of NADPH	6 μM	10 μM
K_i of nitrite[a]	70 μM	50 μM
K_i of $NADP^+$[b]	175 μM	85 μM
NADPH/FAD pattern	intersecting[d]	intersecting
NADPH/nitrate pattern	intersecting[c]	parallel
FAD/nitrate pattern	intersecting[e]	intersecting
Nitrite/NADPH pattern	uncompetitive[f]	uncompetitive
$NADP^+$/nitrate pattern	noncompetitive[g]	uncompetitive

[a] Calculated from the competitive nitrite/nitrate plot at 22 μM FAD and 184 μM NADPH.
[b] Calculated from the competitive $NADP^+$/NADPH plot at 22 μM FAD and 10 mM nitrate.
[c] Result at 22 μM FAD (Figure 1). [d] Result at 10 mM nitrate (Figure 2).
[e] Result at 170 μM NADPH (Figure 3). [f] At 100 μM nitrate and 5 μM or 22 μM FAD.
[g] At 22 μM FAD and 38 μM NADPH.

k_{on}. (c) When the purified enzyme (extensively dialyzed at 5°C) is added to a reaction mixture at 25°C containing saturating concentrations of NADPH and nitrate (but no added FAD), we observe an immediate burst of NADPH oxidation with concomitant nitrite formation (unpublished results). The same burst of activity is observed when the reaction is started by adding NADPH to a mixture of enzyme plus nitrate. But if the reaction is started by adding nitrate to a mixture of enzyme preincubated with NADPH, no burst activity is observed. The burst activity does not persist; the rate decays with a half-time of about 1 minute. The extent and duration of this activity are both too great to stem from a single turnover. We believe that the enzyme (at least as we purify it) contains tightly-bound FAD, perhaps captured during elution from the FAD affinity column (4). If the decay in burst activity results from the dissociation of FAD into a solution of zero FAD concentration, then k_{off} is

about 0.01 sec^{-1}, a rather small value for a first-order substrate dissociation rate constant. But it is likely that k_{off} for FAD is even smaller than 0.01 sec^{-1} and that the observed decay in burst activity actually results from $FADH_2$ dissociation (i.e., once during every 3.8×10^4 catalytic cycles, $FADH_2$ is lost before it can pass electrons on to the next carrier). Thus, NADPH in the absence of nitrate would promote flavin dissociation (as $FADH_2$), while nitrate would retard flavin dissociation by maintaining the bound coenzyme in the oxidized state. The burst activity was not observed at pH 6.18, again suggesting that k_{off} for the flavin increases as the pH is reduced.

The results reported here eliminate the need to postulate a second "downstream" FAD site (4). However, the basic features of the two-site model may still apply. Only instead of being hybrid ping pong-rapid equilibrium random, the reaction sequence can be described as hybrid ping pong-steady state random, which simply means that (a) $NADP^+$ can (but does not have to) dissociate from site 1 after the enzyme is reduced, but before nitrate is reduced at site 2, and (b) the intramolecular electron transfer from the NADPH/FAD site (site 1) to the nitrate site (site 2) is not rate limiting.

ACKNOWLEDGMENTS

The research described in this paper was supported by U.S.P.H.S. Grant GM 26728. Norman D. Schmidt is a recipient of the University of California President's Undergraduate Fellowship.

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