PSEUDOMONAS AERUGINOSA TRANSHYDROGENASE : AFFINITY OF SUBSTRATES FOR THE REGULATORY SITE AND POSSIBLE HYSTERETIC BEHAVIOR

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SUMMARY

The polymeric enzyme transhydrogenase from *Pseudomonas aeruginosa* (NADPH:NAD^+ oxidoreductase; EC 1.6.1.1) has been shown to possess distinct catalytic and regulatory sites, despite the close structural relationship between substrates and effectors. The present report substantiates the previous conclusions from kinetic and affinity chromatography studies, which have suggested that the substrates NADPH and oxidized thionicotinamide adenine dinucleotide phosphate could bind to both catalytic and regulatory sites. In addition, the allosteric R form of the enzyme appears now to be stabilized against high dilution inactivation.

The oxidized substrate thionicotinamide adenine dinucleotide forms a dead end complex on binding to the catalytic site in the T form. The process is slow, and can be termed hysteretic, as defined by Frieden (J. Biol. Chem. (1970), *245,* 5788–5799).

The physiological role of the enzyme transhydrogenase from *Pseudomonas aeruginosa* is to promote NADPH oxidation, with a concomitant generation of NADH. The reverse reaction occurs at a negligible rate, although due to the thermodynamic parameters the reaction should be freely reversible. Kinetic studies have shown that the enzyme is allosterically regulated, and that the Monod–Wyman–Changeux model (1) can be used as a conceptual framework to explore its regulatory properties (2). In the absence of any allosteric ligand the nonfunctional T form is predominant, and prone to spontaneous random aggregation. The active R form is favored by NADPH, whereas NADH is unable to alter the allosteric equilibrium. The potential substrate $NADP^+$ acts as an allosteric inhibitor favoring the T form, while 2'-AMP and several other 2'-phosphate nucleotides activate the enzyme by favoring the R form.

The native isolated form of PATH[1] is characterized by an $s_{20,w}$ of 121 and higher (aggregated polydisperse structure; (3,4). Active enzyme ultracentrifugation studies have shown that disaggregation into a homoge-

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[1] Abbreviations: PATH, *Pseudomonas aeruginosa* transhydrogenase; (TN)AD^+ and (TN)ADP^+, thionicotinamide analogs of NAD^+ and $NADP^+$; (TN)ADH and (TN)ADPH, reduced (TN)AD^+ and (TN)ADP^+.

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neous 34 S population is a prerequisite to enzymic activity (5). This 34 S form (1.6×10^6 molecular weight) corresponds to a polymeric structure of 30 identical protomers of 54,000 daltons (6). All the available evidence suggests that there is a single catalytic site per protomer, successively occupied by the reduced and oxidized coenzyme forms (7), in agreement with the ping pong mechanism indicated by earlier studies (8). Enzyme–bound FAD participates in the reaction (3,9). It appears also that there could be a single regulatory site per protomer for both activation and inhibition, alternatively occupied in the R or T state by 2'-AMP or $NADP^+$, respectively (2)[2].

The enzyme loses activity when highly diluted. In the absence of stabilizing agents, the loss of activity is almost complete after 150 hrs at room temperature (10). FAD and mercaptoethanol afford an efficient protection against the inactivation process. This process has now been reinvestigated, with the aim to find possible protective effects exerted by substrates and effectors. The present findings on these effects give further insights into the regulatory properties of the enzyme.

MATERIALS AND METHODS

NADPH, $(TN)AD^+$, $(TN)ADP^+$ and 2'-AMP were obtained from P–L Biochemicals. PATH was purified as described previously (9).

The enzyme was preincubated under assay conditions, in 0.1 M Tris buffer (pH 7.5), alternatively with each substrate (NADPH–$(TN)AD^+$ and NADPH–$(TN)ADP^+$ reactions), in the presence or absence of the allosteric activator 2'-AMP. For each experiment, addition of the missing reactant (one third of the final volume) initiated the reaction. This procedure also allowed preincubation to be performed in the absence of any substrate, as a control. A preincubation duration of 16 min was sufficient to elicit significant differences in the behavior of the enzyme, as affected by the various experimental conditions. During preincubation, the enzyme was at a concentration of 0.01 unit/ml, as defined by the standard assay (3), corresponding to 2.2 nM of the 34 S species. The final concentrations were 0.1 mM for each substrate, 0.5 mM for 2'-AMP, and 1.45 nM for the enzyme.

All assays were performed at 25°C, using a Perkin Elmer Model 46 spectrophotometer. The reactions were monitored at 398 nm, measuring the formation of (TN)ADH or (TN)ADPH (initial rates). A molar extinction coefficient of 11.3×10^3 was assumed for these reduced coenzyme analogs (11).

RESULTS AND DISCUSSION

The results (Fig. 1) can be explained as follows:

1. The allosteric activator 2'-AMP fully protects the enzyme against high dilution inactivation.
2. This protective effect is depressed when $(TN)ADP^+$ or NADPH are present concurrently with 2'-AMP in the preincubation medium.
3. In the absence of 2'-AMP, $(TN)ADP^+$ and NADPH moderately protect the enzyme.

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[2] In the presence of saturating amounts of 2'-AMP, $NADP^+$ can bind to the catalytic site as well, and function as a substrate. Because of its structural relationship with NADPH and $NADP^+$, 2'-AMP can also bind to the catalytic site (when used at concentrations exceeding 1 mM). The nucleotide competes then with the substrates (2).

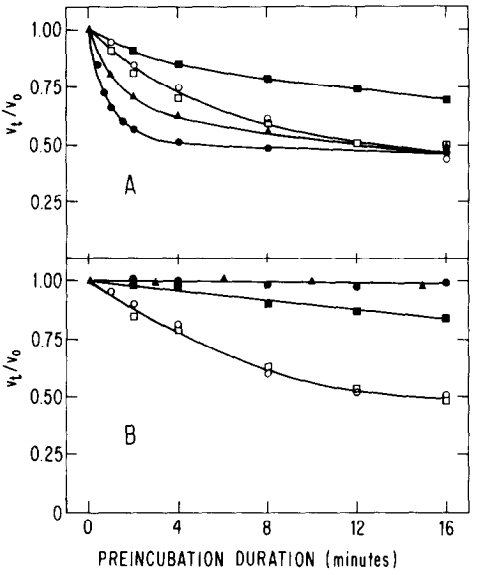

Figure 1: Preincubation of PATH with oxidized and reduced substrates.
A. In the absence of 2'-AMP B. In the presence of 0.75 mM 2'-AMP

v_0 : initial rate without preincubation
v_t : initial rate after the given preincubation duration

■——■ NADPH–(TN)ADP$^+$ reaction, preincubation with (TN)ADP$^+$
○----○ NADPH–(TN)AD$^+$ reaction, preincubation with NADPH
□——□ NADPH–(TN)ADP$^+$ reaction, preincubation with NADPH
●----● NADPH–(TN)AD$^+$ reaction, preincubation with (TN)AD$^+$
▲——▲ control preincubation without any substrate (NADPH–(TN)AD$^+$ reaction).

4. Preincubation with (TN)AD$^+$ results in a significant loss of activity, which exceeds high dilution inactivation

The significance of the 50% loss of activity which has been observed in six different experiments after the 16 min preincubation is not clear.

Addition of 2'-AMP shifts the conformational equilibrium in favor of the R form. This results in an increase of the enzyme–bound FAD fluorescence, and was correlated with activation of the enzyme, as well as with disaggregation into the homogeneous 34 S species (2,3,4,5). In addition it now appears (see Fig. 1B) that stabilization in the R form efficiently prevents high dilution inactivation.

The coenzyme analog (TN)ADP$^+$ has been recognized as an allosteric activator favoring the R form (2), and this correlates with the present results, which indicate that a significant protective effect is afforded to PATH by this nucleotide (Fig. 1A). In view of the interference of (TN)ADP$^+$ with activation by 2'-AMP, it

has been concluded that both compounds are competing for the same effector site (2)[3]. This assumption is now substantiated by the fact that (TN)ADP^+ also interferes with the 2'-AMP-promoted protection of the enzyme (Fig. 1B).

NADPH might possibly bind to both catalytic and effector sites, owing to its close structural relationship with 2'-AMP. The kinetic results gathered to date neither rule out nor ascertain this possibility, which is neither predicted nor opposed by the Monod–Wyman–Changeux model. On the other hand, recent studies on the affinity chromatography behavior of PATH (6,12) have shown that NADPH does have a significant affinity for the regulatory site. Indeed, this might provide a consistent explanation for the results on Fig. 1. As expected, the enzyme is partially protected by NADPH (Fig. 1A), but this allosteric ligand also significantly antagonizes the protective effect of 2'-AMP (Fig. 1B). It would be difficult to rationalize how the binding of NADPH to its separate catalytic site only could prevent full stabilization of the R form by 2'-AMP, in contradiction with all previous kinetic results.

PATH is under the direct allosteric control of the product $NADP^+$. This ensures compliance with the physiological necessity of unidirectional catalysis (5). Therefore, some structural similarity must exist between the catalytic and regulatory sites. This explains why several substrates and substrate analogs bind to the regulatory site, as indicated again by the present preincubation experiments, whereas effectors display some affinity for the catalytic site. PATH therefore contradicts the fairly general rule according to which allosteric effectors do not have any direct chemical or metabolic relation with the substrates and products of the enzymes they act upon (13).

For bireactant single–site enzymes (ping pong mechanism), substrate inhibition by high levels of the second substrate can be explained by a competition between both substrates for the catalytic site in its primary state (14). Such a mechanism takes place for PATH, as indicated by the double reciprocal plots pertaining to the NADPH–(TN)AD^+ reaction (8,10). High levels of the oxidized substrate (TN)AD^+ depress the initial rates as soon as the [(TN)AD^+] / [NADPH] ratio exceeds 3. This substrate inhibition is suppressed in the presence of 0.5 mM 2'-AMP, with [(TN)AD^+] / [NADPH] ratios as high as 40 (10), because stabilization of the R form apparently precludes the abortive binding of (TN)AD^+ to the oxidized substrate site. This means that such a binding occurs preferentially for the T form, thus impeding the NADPH–induced T→R transition.

The results on Fig. 1 are in agreement with the above conclusions. In the absence of any allosteric ligand

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[3] (TN)ADP^+ also functions as a substrate on binding to the catalytic site (in the second stage of the ping pong reaction), but the allosteric equilibrium is not shifted by such a binding (2).

the T form of the enzyme is predominant (2), and preincubation with $(TN)AD^+$ results therefore in the abortive binding of this nucleotide to the catalytic site. The reaction is slow, but the loss of activity is more rapid than if due solely to high dilution inactivation (lowest curve on Fig. 1A). The curve represents relative initial rates, and therefore also indicates that the breakdown of the dead end complex is slow. In the presence of 2'-AMP, the effect of $(TN)AD^+$ is abolished[4].

Enzymes which respond slowly to rapid changes in ligand concentrations (by isomerization and/or displacement of a tightly bound ligand) have been termed hysteretic by Frieden (15). PATH appears to correspond to this definition, with respect to its interaction with $(TN)AD^+$. The frequent occurrence of hysteretic enzymes at critical branch points in metabolic pathways suggests that they have important regulatory roles (15), as exemplified by human erythrocyte pyruvate kinase (16). Preincubation of this enzyme with its substrate ADP results in substrate inhibition, which is slowly relieved on addition of the other substrate phosphoenolpyruvate. The allosteric effector fructose–1,6–diphosphate antagonizes substrate inhibition by ADP. The hysteretic response of the enzyme is assumed to have physiological implications for the glycolytic pathway (16). For PATH, further exploration of the hysteretic mechanism will need additional experiments with NAD^+ as well as NAD^+ analogs. The enzyme has been assumed to act as an essential link between carbohydrate catabolism and the respiratory chain (5). An *in vivo* hysteretic inhibition by NAD^+ would possibly result in a transient diversion of NADPH for biosynthetic purposes.

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[4] In terms of the allosteric model, preferential binding of $(TN)AD^+$ to the T form would mean that a (slow) R→T transition is promoted by this nucleotide. Such a transition has been unnoticed until now, because PATH kinetic assays were generally initiated by addition of the enzyme. Under such conditions, substrate inhibition could be detected for high concentrations of $(TN)AD^+$ only (8,10), and could not be interpreted as an allosteric effect. It is of interest to note now that a $(TN)AD^+$– promoted R→T transition might explain the increasing substrate inhibition by $(TN)AD^+$ observed for the 2'-AMP–activated NADH–$(TN)AD^+$ reaction, upon addition of increasing amounts of $NADP^+$ (8).

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