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Regulatory Properties of the Pyridine Nucleotide Transhydrogenase from *Pseudomonas aeruginosa.* Kinetic Studies and Fluorescence Titration†

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ABSTRACT: Mechanisms involved in the action of the pyridine nucleotide transhydrogenase from *Pseudomonas aeruginosa* (EC 1.6.1.1) have been investigated by means of kinetic studies and fluorescence titration. Our results, as well as those from previous investigations, suggest that the allosteric MWC model (Monod, J., Wyman, J., and Changeux, J. P. (1965), *J. Mol. Biol. 12,* 88–118) may be used as a first step for the explanation of the properties of the transhydrogenase. The basic reaction of the enzyme is the oxidation of reduced triphosphopyridine nucleotide (TPNH) by diphosphopyridine nucleotide (DPN^+). In terms of the model, the functional R state is favored by TPNH, whereas the product triphosphopyridine nucleotide (TPN^+) behaves as an allosteric inhibitor, and is therefore assumed to favor the nonfunctional T state. To a slight extent, the T state is also favored by inorganic phosphate. On the other hand, adenosine 2′-monophosphate and several other 2′-phosphate nucleotides function as activators, and hence are presumed to shift the allosteric equilibrium toward the R state. The studies in this paper suggest a specific regulatory site for the transhydrogenase.

In 1952 an enzyme possessing pyridine nucleotide transhydrogenase activity was discovered in a strain of *Pseudomonas* (Colowick et al., 1952). This enzyme has recently been crystallized from extracts of *Pseudomonas aeruginosa,* and it has been possible to carry out extensive studies on its kinetic and physical properties (Cohen, 1967; Cohen and Kaplan, 1970a,b; Louie and Kaplan, 1970; Louie et al., 1972).

It has been demonstrated that PATH[1] catalyzes the following reaction:

$$TPNH + DPN^+ \rightarrow TPN^+ + DPNH \qquad (1)$$

The reverse reaction occurs at a negligible rate, although from thermodynamic parameters the reaction should be freely reversible. The apparent irreversibility is due to the fact that TPN^+ is a very efficient inhibitor of the enzyme. This also explains why the forward reaction does not reach equilibrium in a reasonable time.

PATH is not highly specific with regard to the pyridine nucleotides and, therefore, several coenzyme analogues can be used as substrates (Kaplan et al., 1952, 1953; Cohen, 1967;

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[1] Abbreviations used are: PATH, *Pseudomonas aeruginosa* pyridine nucleotide transhydrogenase; DPN^+, TPN^+, di- and oxidized triphosphopyridine nucleotide; DPNH, TPNH, reduced DPN and TPN; FAD, flavin adenine dinucleotide; IMP, inosine 5′-monophosphate; AMP, ADP, adenosine mono- and diphosphates; $(AcPy)TPN^+$, 3-acetylpyridine analogue of TPN^+; $deaDPN^+$, and $deaTPN^+$, hypoxanthine analogues of DPN^+ and TPN^+; $(TN)DPN^+$ and $(TN)TPN^+$, thionicotinamide analogues of DPN^+ and TPN^+; ϵ-2′-AMP and ϵ-TPN^+, 1,N^6-ethenoadenine derivatives of 2′-AMP and TPN^+; Tris, tris(hydroxymethyl)aminomethane.

Cohen and Kaplan, 1970a). The absence of definite specificity also allows reactions to occur between two tri- or two diphosphopyridine nucleotides ("T-T" or "D-D" reactions).

2′-AMP acts as an activator of PATH; the following findings have been obtained with this nucleotide: (a) it activates the forward reaction 1 and suppresses its inhibition by the product TPN^+; (b) it allows the reverse of reaction 1 to occur at a significant rate; (c) furthermore, 2′-AMP is needed in order to have a significant rate for each reaction where the reducing substrate is a diphosphopyridine nucleotide and for D-D reactions in general (Kaplan et al., 1953; Cohen and Kaplan, 1970a).

The enzyme is a flavoprotein, and FAD participates in the hydrogen transfer, which occurs between the B sides of both nucleotides (Cohen and Kaplan, 1970a; Louie and Kaplan, 1970). Furthermore, it has been shown that the reaction mechanism is ping-pong bi-bi, according to the nomenclature of Cleland (1963), the reduced substrate being the first substrate to be bound (Cohen, 1967; Cohen and Kaplan, 1970b). All the available evidence suggests that there is a single substrate site per protomer, successively occupied by the reduced and oxidized coenzyme forms (Kaplan, 1972).

It has been found that both 2′-AMP and TPN^+ promote a dramatic change of structure for PATH (Cohen and Kaplan, 1970a). In its native isolated form the enzyme is not homogeneous, and the $s_{20,w}$ value of its main component is 121 S; in the presence of 1 mM 2′-AMP or 1 mM TPN^+ this value is reduced to 33.8 S and the ultracentrifugation pattern is homogeneous. Electron micrographs in the presence of 1 M 2′-AMP indicate that addition of this nucleotide transforms the enzyme structure from a filamentous form into an exclusive population of smaller cylindrical units (Louie et al., 1972). The molecular weight of this 2′-AMP-induced structure has been calculated to be approximately 900 000, with one FAD for about 40 000 (i.e., 20-24 FAD's/molecule). This value would correspond to a monomer molecular weight close to that found in many dehydrogenases.

This report describes the further investigation of the regulatory properties of PATH by means of kinetic studies and fluorescence titration. The MWC model for allosteric transitions (Monod et al., 1965) can be used as a framework to explain our present results as well as those obtained in earlier work.

Materials and Methods

Chemicals. TPN^+, TPNH, (TN)DPN^+, 2′-AMP, 3′-AMP, and 5′-AMP were obtained from P-L Biochemicals; 3′,5′-cyclic AMP from Cyclo Chemical. Glucose 6-phosphate was purchased from Calbiochem. The other chemicals were synthesized in this laboratory: ε-2′-AMP and ε-TPN^+ were prepared by Dr. C. Y. Lee according to the method of Barrio et al. (1972); 2′-IMP and deaTPN^+ were prepared by Mr. F. E. Stolzenbach according to the procedure of Kaplan (1957); (TN)TPN^+ was prepared according to the method of Walter and Rubin (1966). ε-TPN^+ and deaTPN^+ were reduced to ε-TPNH and deaTPNH, using glucose 6-phosphate and glucose-6-phosphate dehydrogenase (Cohen, 1967), and used immediately for the described experiments.

Enzymes. PATH has been purified according to the method of Louis and Kaplan (1970), and was kept in 0.1 M phosphate buffer (pH 7.5) at 4 °C, after dialysis against 10 mM 2-mercaptoethanol and 0.1 mM FAD for stabilization. Unless otherwise indicated, a final concentration of 0.025 enzyme unit/ml was used for every kinetic experiment, as defined by the standard assay (Cohen and Kaplan, 1970a). This concentration corresponded to 10 nM of the 900 000 molecular-weight species, or to 9 μg/ml.

Glucose-6-phosphate dehydrogenase was obtained from Calbiochem.

PATH Initial Rates Determinations. All assays were performed at 25 °C in 1-ml cuvettes with a 1-cm light path, and were initiated by addition of the enzyme. In each case, the oxidized substrate was a thionicotinamide analogue and, therefore, the reactions were always monitored at 398 nm, measuring the formation of (TN)DPNH or (TN)TPNH. A molar extinction coefficient of 11.3×10^3 was assumed for these reduced coenzyme analogues (P-L Biochemicals, 1965). All assays were performed with a Perkin Elmer Model 46 spectrophotometer, equipped with a Perkin Elmer Model 165 recorder.

Fluorescence. FAD fluorescence measurements were performed at 25 °C on an AMINCO Model SPF 125 fluorometer. The excitation wavelength was 365 nm and the fluorescence emission was monitored at 525 nm.

TABLE I: Comparison between Corresponding 2′-AMP and TPNH Analogues.

Addition[a]	Initial Rate (Arbitrary Units)	Reduced Substrate[b]	Initial Rate (Arbitrary Units)
None	1.0	TPNH	100
2′-AMP	66		
2′-IMP or ε-2′-AMP	1.5	ε-TPNH	41
2′-AMP + 2′-IMP	66		
2′-AMP + ε-2′-AMP	66	deaTPNH	29

[a] Reactions for the activation by 2′-AMP: DPNH + (TN)DPN^+ → DPN^+ + (TN)DPNH. Substrates concentration: 0.1 mM; 2′-AMP and 2′-AMP analogue concentration: 0.5 mM; 0.1 M phosphate buffer (pH 7.5). [b] Reactions for the reduced triphosphopyridine nucleotides. Oxidized substrate: (TN)DPN^+; substrate concentration: 0.1 mM; 0.1 M phosphate buffer (pH 7.5).

Results

Distinction between Activator and Substrate Specificities. The structure of 2′-AMP is identical with a portion of the TPNH molecule and, therefore, an important question is whether activation of PATH by 2′-AMP is exerted at a distinct activator site or at the substrate site (Cohen and Kaplan, 1970b). It is of interest that the bulk of experimental evidence indicates that PATH is strictly specific for the 2′-AMP activator, i.e., the 2′-phosphate group and the intact adenine moiety are required to promote activation (Kaplan et al., 1953; Cohen, 1967; Cohen and Kaplan, 1970b). On the other hand, the enzyme does show a significant activity with a number of coenzyme analogues (see Introduction). Such a discrepancy between the activator and substrate specificities suggests that 2′-AMP might bind at a distinct nonsubstrate activator site.

In order to further clarify this problem, experiments were performed with corresponding 2′-AMP and TPNH analogues, which had not been tested previously. These compounds are the inosinic analogues (2′-IMP and deaTPNH) and the fluorescent analogues (ε-2′-AMP and ε-TPNH). The results are summarized in Table I.

The very small activation promoted by the 2′-AMP analogues might be due to 2′-AMP impurities in the analogue preparations. It is therefore reasonable to conclude that ε-2′-AMP and 2′-IMP are unable to activate the enzyme.

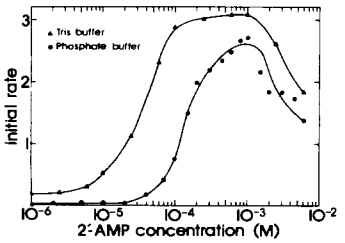

FIGURE 1: Initial rates of the DPNH-(TN)DPN$^+$ reaction (ordinate 1 corresponds to 10 nmol/min) as a function of 2'-AMP concentration, in 0.1 M Tris or phosphate buffer (pH 7.5). Substrate concentrations: 0.1 mM; enzyme concentration: 0.025 units/ml. The initial rates found in the absence of 2'-AMP were not significantly different from those with 1 μM 2'-AMP. Hill coefficient in Tris buffer: 1.8; in phosphate buffer: 2.0.

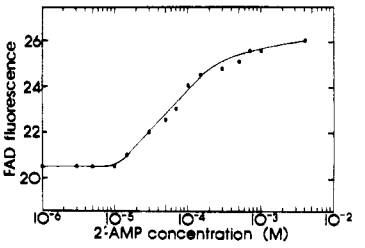

FIGURE 2: FAD fluorescence titration (arbitrary units) as a function of 2'-AMP concentration, in 0.1 M phosphate buffer (pH 7.5). The fluorescence intensity level found in the absence of 2'-AMP was not significantly different from that with 1 μM 2'-AMP.

Furthermore, it is obvious that they do not compete with 2'-AMP. On the other hand, the corresponding adenine substituted TPNH analogues are significantly active as reduced substrates for PATH. These results are consistent with the assumption that 2'-AMP might bind at a distinct activator site rather than at the substrate (or catalytic) site.[2]

2'-AMP-Activated DPNH-(TN)DPN$^+$ Reaction. Until the present time, the DPNH-(TN)DPN$^+$ reaction has never been investigated in detail and has been considered to be almost completely dependent on 2'-AMP (Kaplan, 1972). A more careful investigation has now shown that the reaction actually proceeds at a very slow rate and that the usual first-order relationship between initial rate and enzyme concentration is obtained. Therefore, it is not correct to state that the enzyme is totally inactive for the DPNH-(TN)DPN$^+$ reaction in the absence of 2'-AMP.

Using a relatively narrow 2'-AMP concentration range, Cohen (1967) reported that the activation curves were not hyperbolic, i.e., the double-reciprocal plots displayed an upward curvature. This problem has now been reinvestigated over a wider range of 2'-AMP concentration, in Tris or phosphate buffers (pH 7.5), and with two different concentrations of enzyme (0.025 and 0.0025 enzyme units/ml). In each case, the activation curve has a sigmoidal shape (direct plot with arithmetical abscissa), but falls off when the activator concentration exceeds 1 mM. Figure 1 shows two typical activation curves (in 0.1 M buffers, logarithmic abscissa) and it can be seen that the activation phenomenon is less pronounced in phosphate buffer than in Tris buffer (preliminary experiments have shown that this is mainly due to the phosphate anions, and not to the metallic cations). However, the maximal rates extrapolated to infinite 2'-AMP concentration are the same in both buffers. It was also found that the activation curve in 0.01 M phosphate buffer is close to the curve in 0.1 M Tris buffer, but that the Hill coefficients are always higher in phosphate buffer than in Tris buffer. Our results lead, therefore, to the tentative conclusion that 2'-AMP binds to PATH in a cooperative way in both buffers and that phosphate anions act as antagonists of 2'-AMP.

Another distinctive feature of the activation curves is the decreased activation when the concentration of 2'-AMP exceeds 1 mM. The fact that 2'-AMP is identical with a portion of the potential substrate TPNH suggests that the activator might compete with DPNH for the substrate site, thereby promoting an apparent decreased activation when used at high concentration. The results given in Table II substantiate this assumption, since such a competition might also explain why 3'-AMP, 5'-AMP, and 3',5'-cyclic AMP similarly act as inhibitors when added in high concentration to the 2'-AMP-activated DPNH-(TN)DPN$^+$ reaction. These mononucleotides have been shown to be ineffective as activators (Louie et al., 1972), but their structural relationships with substrates presumably allow them, as well as 2'-AMP, to have some affinity for the substrate site, which lacks strict specificity.

TABLE II: Effect of High Concentration of Adenosine Monophosphate Isomers on the DPNH-(TN)DPN$^+$ Reaction.[a]

Addition	Initial Rate (Arbitrary Units)
2'-AMP (1 mM)	100
2'-AMP (5 mM)	65
2'-AMP (1 mM) + 3'-AMP (4 mM)	70
2'-AMP (1 mM) + 5'-AMP (4 mM)	67
2'-AMP (1 mM) + 3',5'-cyclic AMP (4 mM)	71

[a] Substrate concentration: 0.1 mM; 0.1 M phosphate buffer (pH 7.5).

Fluorescence Titration. Louie et al. (1972) have demonstrated that the enzyme-bound FAD fluorescence is enhanced fourfold upon addition of 1 mM 2'-AMP, with a concomitant alteration of the structure of PATH. However, in these studies, no determinations were carried out on the effect of subsaturating levels of 2'-AMP. It was therefore of interest to check this fluorescence enhancement by using the same 2'-AMP concentration range as that used for the enzyme activation curves. The results are shown on Figure 2 (logarithmic abscissa). The relatively high background fluorescence intensity is caused by the free dialyzed FAD (see Materials and Methods).

Figure 2 indicates that up to a concentration of 10 μM 2'-AMP, the fluorescence intensity of FAD is not significantly changed. With higher concentrations, there is strong evidence for structural alteration, since the fluorescence intensity is significantly increased. The FAD fluorescence curve of Figure 2 strongly resembles the lower activation curve of Figure 1 (but without "excess inhibition" when the 2'-AMP concentration exceeds 1 mM). It is therefore possible to correlate changes of catalytic activity with the induced structural alteration over a wide 2'-AMP concentration range (up to 1 mM), and not only for saturating concentrations of this nucleotide. The obvious decrease in enzyme activity seen with 2'-AMP concentrations exceeding 1 mM (see Figure 1) does not correspond

[2] Our present results are closely related to those reported recently by Dieter et al. (1974) for glutamate dehydrogenase. ϵ-DPNH is fully active as a coenzyme for the given dehydrogenase, indicating low specificity of the coenzyme binding the site. ADP usually activates the enzyme upon binding to a distinct effector site, whereas ϵ-ADP does not affect the activity, indicating a stricter specificity of the effector site.

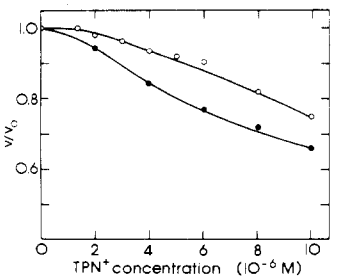

FIGURE 3: Inhibition by TPN^+ of the TPNH-(TN)DPN^+ reaction in 0.1 M Tris buffer (pH 7.5). (TN)DPN^+ concentration: 0.1 mM; TPNH concentration: upper curve 0.1 mM, lower curve 30 μM; v: initial rate for a given inhibitor concentration; v_0: initial rate in the absence of inhibitor.

to any notable variation of the enzyme-bound FAD fluorescence and is, thus, apparently not due to any significant structural change that could affect FAD (and hence the catalytic site).

The FAD fluorescence intensity level obtained with a 2′-AMP concentration of 1 mM and upwards presumably corresponds to the homogeneous population of disaggregated units seen by electron microscopy with the same activator concentration (Louie et al., 1972). For the concentration range from 10 μM to 1 mM, the shape of the fluorescence titration curve suggests that the transition process induced by 2′-AMP is gradual. This indicates that with subsaturating levels of 2′-AMP both long rods and smaller cylindrical units of 900 000 molecular weight might be found.

Inhibition by TPN^+. For the 2′-AMP-activated DPNH-(TN)DPN^+ reaction, the product inhibition pattern is consistent with a ping-pong bi-bi mechanism (Cohen, 1967; Cohen and Kaplan, 1970b). For the same reaction TPN^+ shows a much greater inhibitory effect than DPN^+; however, TPN^+ does not behave as expected as an alternative substrate. This failure to comply with the usual product inhibition pattern suggests that inhibition by TPN^+ is due not to the binding at the substrate site but perhaps at a hypothetical effector site.

A thorough study of the mechanism of inhibition by TPN^+ is not possible, since TPN^+ acts both as an inhibitor as well as a potential substrate. Nevertheless, we have tried to obtain information for the concentration range between 1 and 10 μM TPN^+ (which is significantly below the inhibition saturation level) with two different TPNH concentrations in the TPNH-(TN)DPN^+ reaction (see Figure 3). In both cases, the curves are not hyperbolic but, on the contrary, show the characteristics indicative of sigmoidal curves. Furthermore, the sigmoidicity is decreased when the TPNH concentration is decreased. These findings suggest that TPN^+ binds in a cooperative way to PATH and that TPNH antagonizes the binding.

As already noted, the TPNH analogues ϵ-TPNH and deaTPNH show significant activities as reduced substrates (see Table I). It was of interest to check the possible inhibition of the TPNH-(TN)DPN^+ reaction by the oxidized forms of these analogues as compared to inhibition by TPN^+. Under the standard assay conditions (Cohen and Kaplan, 1970a), 5 μM ϵ-TPN or deaTPN$^+$ have no detectable inhibitory effect, whereas TPN^+ promotes a significant inhibition at that concentration (~20%). When the concentration is increased above 50 μM, inhibition by TPN^+ exceeds 50%, whereas ϵ-TPN^+ and deaTPN$^+$ promote only a slight inhibition (<5%). This small inhibitory effect could be due to TPN^+ impurities in the TPN^+ analogues. Another reason for this apparent inhibition could also be that at the concentrations employed the TPNH-ϵ-TPN^+ or TPNH-deaTPN$^+$ reaction is no longer negligible, and hence can be in competition with the reduction of (TN)DPN^+. A hydrogen transfer between two triphosphopyridine nucleotides is a known reaction of PATH (Kaplan et al., 1953) and does not require activation by 2′-AMP (except in the special case of the reduction of TPN^+, in which 2′-AMP is necessary to antagonize inhibition of PATH by TPN^+).

It appears, therefore, from our results that the inhibition specificity corresponds to the activation specificity, since ϵ-TPN^+ and deaTPN$^+$ correspond to the 2′-AMP analogues ϵ-2′-AMP and 2′-IMP, which have been found to produce no activation effect (see Table I). The 2′-phosphate group and the intact adenine moiety apparently are essential for both activation and inhibition.

Substrate and Effector Behavior of (TN)TPN^+. PATH does not exhibit a significant activity when the reduced substrate is a diphosphopyridine nucleotide, in which the 2′-phosphate group is missing (Kaplan et al., 1953; Cohen, 1967; Cohen and Kaplan, 1970a; Kaplan, 1972). The oxidation of DPNH by TPN^+ is a special case and has been discussed in the preceding section. The oxidation of DPNH by (TN)DPN^+, deaDPN$^+$, or NMN^+ is a more general case, since none of these oxidized nucleotides behaves as an inhibitor of PATH. However, 2′-AMP is needed in order to have a significant oxidation of DPNH. The oxidation of DPNH by (TN)TPN^+ is an exception, because it proceeds at a significant rate without any activator (Cohen, 1967). Furthermore, it appears that the reaction is only moderately sensitive to activation by 2′-AMP. It was therefore of interest to obtain further insight into some reactions in which (TN)TPN^+ is the oxidized substrate.

Table III compares the relative activities when DPNH or TPNH are hydrogen donors with (TN)DPN^+ or (TN)TPN^+ as acceptors. The effects of TPN^+ and 2′-AMP on these reactions are also included. The contrast in activities between (TN)DPN^+ and (TN)TPN^+ as acceptors when DPNH is the donor is striking (comparison between lines 1′ and 2′ on Table III). On the other hand, the comparison between lines 1 and 2 of Table III indicates that (TN)TPN^+ is a less effective oxidized substrate than (TN)DPN^+, when used with TPNH as hydrogen donor. It is also worth noting that activation by 2′-AMP is antagonized when (TN)TPN^+ is used instead of (TN)DPN^+ (lines 4 and 4′ compared to lines 3 and 3′), but that some protection is afforded against inhibition by TPN^+ (lines 6 and 6′ compared to lines 5 and 5′).

An explanation of why the DPNH-(TN)TPN^+ reaction proceeds at a significant rate in the absence of 2′-AMP is to assume that (TN)TPN^+ is able to activate PATH (as does 2′-AMP), in addition to its role as a substrate (this could also explain why some protection is afforded against inhibition by TPN^+). (TN)TPN^+ can be considered as containing an intact 2′-AMP molecule, so that the activation specificity would be obeyed (2′-phosphate group and intact adenine moiety). One is led to the conclusion hat (TN)TPN^+ and 2′-AMP might compete for the same activator site. This assumption is substantiated by our finding that (TN)TPN^+ antagonizes activation by 2′-AMP.

Discussion

The most remarkable reactional characteristics of PATH are the non-Michaelian kinetics often exhibited by the enzyme, the apparent irreversibility of the TPNH-DPN^+ reaction, the antagonistic behavior of 2′-AMP and TPN^+, and the dependence of 2′-AMP affecting D-D reactions. No attempt has previously been made to interpret and relate all these properties

TABLE III: Comparison between (TN)DPN$^+$ and (TN)TPN$^+$ Used as Oxidized Substrates, with DPNH or TPNH as Reduced Substrates, in the Presence or Absence of Effectors, in 0.1 M Tris Buffer (pH 7.5)

Reduced Substrate (50 μM)	Oxidized Substrate (0.1 mM)	Effector Added	Activity (Arbitrary Units)	% Change on Effector Addition
1. TPNH	(TN)DPN$^+$	None	100	
2. TPNH	(TN)TPN$^+$	None	72	
3. TPNH	(TN)DPN$^+$	2′-AMP (0.5 mM)	126	+26
4. TPNH	(TN)TPN$^+$	2′-AMP (0.5 mM)	81	+12.5
5. TPNH	(TN)DPN$^+$	TPN$^+$ (10 μM)	72	−28
6. TPNH	(TN)TPN$^+$	TPN$^+$ (10 μM)	63	−12.5
1′. DPNH	(TN)DPN$^+$	None	6.1	
2′. DPNH	(TN)TPN$^+$	None	34	
3′. DPNH	(TN)DPN$^+$	2′-AMP (0.5 mM)	72	+1100
4′. DPNH	(TN)TPN$^+$	2′-AMP (0.5 mM)	41	+21
5′. DPNH	(TN)DPN$^+$	TPN$^+$ (10 μM)	3.8	−38
6′. DPNH	(TN)TPN$^+$	TPN$^+$ (10 μM)	26	−24

in terms of an allosteric model. It is evident that the fitting of the available experimental data with any model can only be qualitative as long as the structure of the enzyme is not known with more detail (for example, the exact number of protomers). Furthermore, there is no qualitative way to distinguish between the two most used allosteric models (except the case of negative homotropic cooperativity of binding; Goldbeter, 1974), i.e., the symmetry (MWC) model (Monod et al., 1965) and the sequential model (Koshland et al., 1966; Kirtley and Koshland, 1967). Since a negative homotropic behavior has never been detected for PATH, either model might be used to account for the experimental results gathered to date. In view of the exceptionally large size of the enzyme, we think, however, that serious difficulties would be encountered should the sequential model be chosen as the first conceptual framework. Therefore, we believe that, for the sake of simplification, the MWC model should be used as a first step in the correlation of the characteristics of PATH with those of well-defined regulatory enzyme mechanisms. It appears, indeed, that this model provides a consistent framework for explaining our results, as well as those from previous investigations.[3]

Using the nomenclature of Monod et al. (1965), one can state the reactional properties of PATH as follows: (1) The reduced substrate TPNH acts as an allosteric homotropic ligand. Its cooperativity is decreased by the activator 2′-AMP and increased by the inhibitor TPN$^+$. This can be deduced from the kinetic results of Cohen (1967) and of Cohen and Kaplan (1970b). (2) The results of this report (inhibition by TPN$^+$) suggest that the inhibitor TPN$^+$ exhibits cooperative homotropic effects and that TPNH increases this cooperativity. (3) The results of Cohen (1967), as well as those of this report (2′-AMP-activated DPNH–(TN)DPN$^+$ reaction), show that the activator 2′-AMP also exhibits cooperative homotropic effects, and that phosphate anions increase this cooperativity. (4) The rate-concentration curves for DPNH and (TN)DPN$^+$, in the presence of different concentrations of 2′-AMP or TPN$^+$, are always hyperbolic (Cohen, 1967; Cohen and Kaplan, 1970b). This can be interpreted as experimental demonstration that DPNH and (TN)DPN$^+$ do not act as allosteric ligands (i.e., they are unable to modulate the catalytic response of the enzyme).

These data can be accounted for by assuming that PATH exists (at least potentially) in two conformational states R and T, and that each of the specific ligands TPNH, 2′-AMP, TPN$^+$, and inorganic phosphate is endowed with differential affinity towards these two states.[4] The R–T equilibrium is shifted in favor of the R state by the substrate TPNH and the activator 2′-AMP, and in favor of the T state by the inhibitors TPN$^+$ and inorganic phosphate. It has been shown that the maximal initial rates (extrapolated to infinite concentration of both substrates) are not significantly different for the TPNH–(TN)DPN$^+$, for the 2′-AMP-activated TPNH–(TN)DPN$^+$, and for the 2′-AMP-activated DPNH–(TN)DPN$^+$ reactions (Cohen and Kaplan, 1970b). Furthermore, varying concentrations of TPN$^+$ do not affect the maximal initial rate (extrapolated to infinite TPNH concentration) when added to the 2′-AMP-activated TPNH–(TN)DPN$^+$ reaction. Finally, this report shows that inorganic phosphate does not affect the maximal initial rate extrapolated from the activation curves (2′-AMP-activated DPNH–(TN)DPN$^+$ reaction). Therefore, PATH must be considered as a "K system" (Monod et al., 1965) with respect to the interactions between the reduced substrate TPNH and the effectors 2′-AMP and TPN$^+$, and with respect to the interaction between 2′-AMP and inorganic phosphate. Such an assumption has already been made by Rydström et al. (1973) with respect to the interaction between TPNH and TPN$^+$.

DPNH is unable to shift the allosteric equilibrium and must therefore be assumed to bind nonpreferentially to both R and T forms. This tentative explanation in terms of the MWC model is supported by the kinetic results of Cohen (1967) and of Cohen and Kaplan (1970b) pertaining to the DPNH–(TN)DPN$^+$ reaction (effects of varying concentrations of TPN$^+$ and/or 2′-AMP on the extrapolated maximal rates and the apparent K_m for DPNH). PATH must therefore be con-

[3] It is thus our view at the present time that PATH substrates and effectors bind at different sites, in spite of their close structural relationships. However, Dr. B. Wermuth in our laboratory has recently undertaken physicochemical studies that might show that a half-site reactivity model could represent for PATH another tentative mechanism, entirely different from the model proposed in this report.

[4] The binding of phosphate anions to PATH would not be uncommon, since other enzymes such as phosphatases (Webb, 1963), creatine kinase (Webb, 1963), ribonuclease A (Anderson et al., 1968), and muscle aldolase (Ginsburg and Mehler, 1966) have been shown to bind inorganic phosphate (a common feature of these enzymes and of PATH is that their substrates contain phosphate groups). For PATH, it is unlikely that inorganic phosphate might have any regulatory effect in vivo, since its affinity for the enzyme appears to be very low.

sidered as a "V system" with respect to the interaction between DPNH and the effectors 2′-AMP or TPN^+, which means that the system behaves differently depending on which reduced substrate is used. 2′-AMP as well as TPN^+ affect the DPNH-(TN)DPN^+ reaction because they favor the R or T form, respectively, and because significant catalytic activity is exhibited for the given substrates by the R form only. The minute activity detected in the absence of 2′-AMP for the DPNH-(TN)DPN^+ reaction is indicative of the small amount of R form existing prior to the binding of a ligand exhibiting differential affinity. The T form is therefore predominant in the absence of any such ligand.

The fact that DPNH does not behave as an allosteric ligand (unlike TPNH) may be related to the absence of the 2′-phosphate group, since the reduction of (TN)DPN^+ by NMNH or deaDPNH (which similarly lack the 2′-phosphate group) also requires 2′-AMP in order to occur at a significant rate (Cohen and Kaplan, 1970b). On the other hand, the dependence of these reactions (reduction of (TN)DPN^+ by NMNH or deaDPNH) on 2′-AMP is in agreement with our assumption that (TN)DPN^+ does not behave as an allosteric ligand either. Unfortunately, the available kinetic data are not yet sufficient to state whether this inability to shift the allosteric equilibrium may be ascribed to nonpreferential binding, as has been assumed for DPNH, or to another phenomenon (this inability does not appear to be a specific property of (TN)DPN^+, since the oxidation of DPNH by NMN^+ or deaDPN$^+$ is also strongly dependent on 2′-AMP; Kaplan et al., 1953). The characteristics of the ping-pong bi-bi mechanism and the fact that PATH appears to have only one single substrate site per protomer might prompt one to assume that the shift of a preexisting allosteric equilibrium is induced only by the substrate promoting the first stage of the enzymatic reaction (i.e., the reduced substrate), or by an effector (activator or inhibitor). This assumption amounts to considering PATH as a unireactant enzyme, as far as the MWC model is concerned, and would account for the kinetic results pertaining to (TN)DPN^+, NMN^+, and deaDPN$^+$.

It has been suggested in this report that (TN)TPN^+ behaves both as an activator and as a substrate, the activation phenomenon being due to the binding at the specific activator site. This would explain why (TN)TPN^+ antagonizes activation by 2′-AMP. On the other hand, there is also a strong indication that inhibition by TPN^+ is induced by the binding of this nucleotide at a nonsubstrate effector site. These results do not contradict our assumption that the binding of an oxidized substrate to the *substrate* site only (in the second stage of the enzymatic reaction) does not promote any allosteric effect. An additional argument along these lines is the fact that (AcPy)-TPN^+ is an efficient activator of PATH, whereas it is a poor substrate (Cohen and Kaplan, 1970b). This activation effect must also be due to the binding at the activator site, since (AcPy)TPN^+ antagonizes activation by 2′-AMP, as does (TN)TPN^+.

The striking similarity between activation and inhibition specificity (intact adenine moiety and 2′-phosphate group requirement) suggests that there might be only one single effector site per protomer for both activation and inhibition, alternatively occupied in the R or T state (this would explain why two TPN^+ analogues behave as activators). The specificities are, nevertheless, not identical. For inhibition, it appears that the oxidized nicotinamide ring must be intact too (i.e., TPN^+ is the only known allosteric inhibitor), whereas for activation this part may be modified, since (AcPy)TPN^+ and (TN)TPN^+ behave as activators. Furthermore, the well-documented activation by 2′-AMP shows that the nicotinamide ring is not even necessary. This is further substantiated by the fact that 2′,5′-ADP and 2′-P-ADPR have also been recognized as activators for PATH (Kaplan et al., 1953). The specificity discrepancy agrees with the fact that activation or inhibition occurs when PATH is stabilized in two different conformations, even if there might be only one single effector site.

The activation specificity is actually not sufficient to ensure a really specific response of PATH, since five compounds unquestionably act as activators. Such an unspecific response might be difficult to reconcile with stringent metabolic requirements. In this connection, one has to point out, however, that (AcPy)TPN^+ and (TN)TPN^+ do not occur in vivo. On the other hand, there are no reports to date that would state the in vivo occurrence of 2′-AMP, 2′,5′-ADP, or 2′-P-ADPR in significant amounts. These compounds can undoubtedly appear by enzymatic breakdown of TPN^+, but they must rapidly undergo further catabolic reactions. We are therefore inclined to think that activation of PATH by 2′-AMP and related nucleotides might be an in vitro characteristic. This would be in agreement with the fact that the most dramatic activation effect is found for D-D reactions, which, as yet, have no known physiological significance. If the activation phenomenon is of no importance in vivo, a separate activator site is indeed unnecessary. This would agree with our assumption that there might be only one single effector site per protomer for both activation and inhibition, alternatively occupied in the R and T state. This is tantamount to saying that activation by several 2′-P-nucleotides must be due to their close structural relationships with TPN^+, and that PATH might act in vivo as a unidirectional catalyst that is favoring the oxidation of TPNH by DPN^+.

Monod et al. (1965), as well as Koshland et al. (1966), assume that allosteric proteins are closed systems of finite extent, characterized by a constant number of protomers. For PATH, the polydisperse structure seen by Louis et al. (1972), with large helical oligomers of nonuniform length, is difficult to reconcile with such an assumption. The following paper in this issue (Widmer and Kaplan, 1976) will be devoted to the investigation of these macromolecular changes.

Added in Proof

After this manuscript was completed, two of our results were confirmed by other authors (Höjeberg and Rydström, 1976). These authors concluded from the affinity chromatography behavior of PATH that the 2′-AMP site (allosteric site) is different from the catalytic site. They also found that titration of the enzyme with 2′-AMP results in a sigmoidal response of the enzyme-bound FAD.

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Regulatory Properties of the Pyridine Nucleotide Transhydrogenase from *Pseudomonas aeruginosa.* Active Enzyme Ultracentrifugation Studies†

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ABSTRACT: Active enzyme ultracentrifugation studies of the pyridine nucleotide transhydrogenase from *Pseudomonas aeruginosa* (EC 1.6.1.1.) show that the enzymatic reaction is catalyzed by a molecular species characterized by an $s_{20,w}$ value of about 34 S, whatever the reduced substrate may be (tri- or diphosphopyridine nucleotide). The filamentous aggregated form of the enzyme ($s_{20,w}$ = 121 S and higher), identified by previous investigations (Cohen, P. T., and Kaplan, N. O. (1970), *J. Biol. Chem. 245,* 2825-2836; Louie, D. D., Kaplan, N. O., and Mc Lean, J. D. (1972), *J. Mol. Biol. 70,* 651-664), appears, therefore, to be an inactive species. The physiological implications of the enzyme are discussed. Several lines of evidence lead to the conclusion that the transhydrogenase might act as an essential link between carbohydrate catabolism and the respiratory chain.

We have reported, in the preceding paper of this issue (Widmer and Kaplan, 1976), that the MWC allosteric model (Monod et al., 1965) might be used as a framework to explore the regulatory characteristics of the enzyme transhydrogenase from *Pseudomonas aeruginosa.* The R state is favored by TPNH,[1] 2′-AMP, and several other 2′-phosphate nucleotides, whereas TPN^+ and inorganic phosphate show more affinity for the T state. On the other hand, the structure of PATH, as elucidated by ultracentrifugation (Cohen, 1967; Cohen and Kaplan, 1970a) and electronmicroscopy (Louie et al., 1972), might prompt one to assume that association-dissociation reactions could characterize the catalytic mechanism of the enzyme. Such a regulatory mechanism would not be in agreement with the fairly general rule that allosteric enzymes have a fixed number of protomers, which is independent of any allosteric transitions which might occur.

The main component of the sedimentation pattern of PATH in its native form is characterized by an $s_{20,w}$ value of 121 S, whereas aggregated material, not actually in solution, sediments with an even higher speed (Cohen and Kaplan, 1970a). These two components should correspond to the rodlike shaped polydisperse structure seen on electronmicrographs of the native enzyme (Louie et al., 1972). On the other hand, Cohen and Kaplan (1970a) found that in the presence of 1 mM 2′-AMP or 1 mM TPN^+ the sedimentation pattern is homogeneous, and characterized by a single component with an $s_{20,w}$ of 33.8 S. This component has been assumed by Louie et al. (1972) to correspond to the uniform population of cylindrical particles (900 000 dalton) seen on electronmicrographs after addition of 1 mM 2′-AMP. A very small amount of such units are already seen on electronmicrographs of the native structure, and should correspond to the component with an $s_{20,w}$ of

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[1] Abbreviation: PATH, *Pseudomonas aeruginosa* pyridine nucleotide transhydrogenase; for other abbreviations, see footnote 1 of the preceding paper in this issue (Widmer and Kaplan, 1976).