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STEADY-STATE KINETICS AND THE INACTIVATION BY 2,3-BUTANEDIONE OF THE ENERGY-INDEPENDENT TRANSHYDROGENASE OF *ESCHERICHIA COLI* CELL MEMBRANES

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Summary

Kinetic measurements indicate that the energy-independent transhydrogenation of 3-acetylpyridine-NAD^+ by NADPH in membranes of *Escherichia coli* follows a rapid equilibrium random bireactant mechanism. Each substrate, although reacting preferentially with its own binding site, is able to interact with the binding site of the other substrate to cause inhibition of enzyme activity. 5′-AMP (and ADP) and 2′-AMP interact with the NAD^+- and $NADP^+$-binding sites, respectively. Phenylglyoxal and 2,3-butanedione in borate buffer inhibit transhydrogenase activity presumably by reacting with arginyl residues. Protection against inhibition by 2,3-butanedione is afforded by $NADP^+$, NAD^+, and high concentrations of NADPH and NADH. Low concentrations of NADPH and NADH increase the rate of inhibition by 2,3-butanedione. Similar effects are observed for the inactivation of the transhydrogenase by tryptic digestion in the presence of these coenzymes. It is concluded that there are at least two conformations of the active site of the transhydrogenase which differ in the extent to which arginyl residues are accessible to exogenous agents such as trypsin and 2,3-butanedione. One conformation is induced by low concentrations of NADH and NADPH. Under these conditions the coenzymes could be reacting at the active site or at an allosteric site. The stimulation of transhydrogenase activity by low concentrations of the NADH is consistent with the latter possibility.

Abbreviations: $APNAD^+$, 3-acetylpyridine-NAD^+; TPCK-trypsin, trypsin treated with L-(tosylamido 2-phenyl)ethyl chloromethyl ketone to inhibit contaminating chymotryptic activity.

Introduction

The membranes of mitochondria and chromatophores, and the cell membrane of certain bacteria, including *E. coli*, catalyze the reversible transfer of hydrogen between the oxidized and reduced forms of NAD^+ and $NADP^+$ [1,2].

$$NADPH + NAD^+ \rightleftharpoons NADP^+ + NADH$$

Input of energy results both in an increase in the rate of the energy-independent reaction as well as an increase in the apparent equilibrium constant from 1 to about 500. Energy appears to be supplied as an energized state formed either by substrate oxidation through the respiratory chain or from ATP by a reversal of the reactions of oxidative phosphorylation. The mechanism of the process which results in an alteration of the kinetic properties of the transhydrogenase is still obscure. Recent results on the reconstitution of the purified enzyme into phospholipid vesicles suggests that the enzyme responds to a transmembrane potential and/or proton gradient [3,4]. It is likely that this results in a conformational change in the enzyme [5,6].

There have been several studies of the kinetic mechanism of the transhydrogenase. Rydström and coworkers [6,7] and Houghton et al. [8] found that the energy-independent transhydrogenation occurring in beef-heart submitochondrial particles and *E. coli* membranes followed a Theorell-Chance mechanism. The same mechanism also occurred in mitochondrial energy-dependent transhydrogenation [6]. In the Theorell-Chance mechanism the addition or release of substrates and products from the enzyme occurs in a definite order and the ternary complex which is formed has a very short life-time. However, in a recent study using a detergent-solubilized preparation from *E. coli*, Hanson [9] concluded, in contrast to the previous workers, that transhydrogenation followed the kinetics of a rapid equilibrium random bireactant mechanism. In this mechanism the addition and release of substrates and products from the enzyme occurs in no fixed order. The rate-limiting step is generally considered to be the interconversion of the ternary enzyme-substrates and ternary enzyme-products complexes. Hanson pointed out that most of the data of the previous workers are also consistent with this type of mechanism but he was able by the use of competitive inhibitors for each substrate to eliminate the ordered in favour of the random mechanism.

The transfer of hydrogen between NAD(H) and NADP(H) catalyzed by the transhydrogenases of mitochondrial and *E. coli* membranes occurs without exchange with the hydrogen atoms of the surrounding water and is stereospecific for the 4A hydrogen of NADH and the 4B hydrogen of NADPH [10]. These results together with kinetic and inhibitor studies suggest that there are separate binding sites for the two substrates on the enzyme. The nature of the groups involved in transhydrogenase activity at these two sites has been studied by chemical modification. Arginyl and cysteinyl residues appear to be involved in the mitochondrial enzyme since modification of the transhydrogenase with 2,3-butanedione, *N*-ethylmaleimide and 5,5′-dithiobis-(2-nitrobenzoic acid) resulted in loss of activity [11—15]. Houghton et al. [16] have studied the effect of *N*-ethylmaleimide on the energy-independent transhydrogenase of *E. coli* membranes. Protection against inhibition was achieved with NAD^+ and

$NADP^+$, but NADPH accelerated the rate of inhibition. These results, which are similar but not identical to those obtained with submitochondrial particles, suggest that thiol groups are present near the NAD^+ and $NADP^+$ binding-sites of the bacterial enzyme. Arginyl residues may also be involved at these sites in the bacterial transhydrogenase although data supporting this suggestion have not been published [16].

In the present paper we have investigated the kinetic mechanism of the energy-independent transhydrogenase in membranes of *E. coli*. In agreement with the conclusions of Hanson [9] for the solubilized enzyme, we propose that this reaction follows a rapid equilibrium random bireactant mechanism. The possible involvement of arginyl residues at the NAD^+- and $NADP^+$-binding sites of the enzyme has been studied using 2,3-butanedione and phenylglyoxal. At least two conformations of the active site of the transhydrogenase, which differ in the extent of their reaction with 2,3-butanedione and with trypsin, have been detected. One conformation is induced by the presence of low concentrations of NADH and NADPH. These coenzymes may be acting at an allosteric site.

Materials and Methods

Growth of bacteria

E. coli ML308-225 or the proline auxotroph *E. coli* W6 (ATCC 25377) were used in the experiments. Both strains were grown in a minimal salts-glucose medium containing 12 μM ferric citrate [17]. The growth medium for *E. coli* W6 was supplemented with 50 μg proline/ml medium. The cells were grown at 37°C with vigorous aeration from a sparger and were harvested in the late exponential phase of growth. The cells of *E. coli* ML 308-225 were washed once with 50 mM Tris-H_2SO_4 buffer, pH 7.8, containing 10 mM $MgCl_2$ (Buffer 1). The cells of *E. coli* W6 were washed with 50 mM Tris-H_2SO_4 buffer (pH 7.8) containing 1 mM EDTA and 1 mM dithiothreitol (Buffer 2). The washed cells were stored at −20°C.

Preparation of membranes

Membranes were prepared as described by Bragg et al. [17]. With the exception of the experiment shown in Fig. 4 (panels 3 and 4) where those from *E. coli* ML308-225 were used, the membranes employed in the experiments were prepared from *E. coli* W6. They were suspended either in Buffer 2 or in 50 mM sodium borate buffer, pH 7.8 (Buffer 3) as indicated in the legends to the figures and tables. The membranes from *E. coli* ML308-225 were taken up in Buffer 1.

Assay of energy-independent transhydrogenase activity

This was based on the method of Kaplan [18]. The reaction was carried out at 20—22°C in a mixture which contained 50 mM potassium phosphate buffer (pH 7.0), 1 mM KCN, 50—275 μg of membrane protein (as indicated in the legends to the figures and tables), 1 mM 3-acetylpyridine-NAD^+ and 0.5 mM NADPH in a final volume of 1 ml. Variations in the concentrations of the components present in the 'standard' assay are indicated in the descriptions of the

individual experiments. The reduction of 3-acetylpyridine-NAD^+ was measured as an increase in the absorbance at 375 nm using a Coleman 124 spectrophotometer. The extinction coefficient of 3-acetylpyridine-NADH was taken as $5.1 \cdot 10^3\ l \cdot mol^{-1} \cdot cm^{-1}$ [18]. Protein was determined by the method of Lowry et al. [19].

Reaction of membranes with 2,3-butanedione, phenylglyoxal and trypsin

The conditions for the individual experiments are described in the legends to the figures and tables. 2,3-Butanedione was redistilled prior to use. It and phenylglyoxal were dissolved in Buffer 3 just prior to carrying out the experiment. Pyridine nucleotide coenzymes and analogues used in the modification experiments were made up in Buffer 3. Experiments involving reduced pyridine nucleotide coenzymes were carried out in the dark to minimize photooxidation of the reduced coenzymes [20]. The final pH of the reaction mixture containing 2,3-butanedione was 7.0—7.2.

TPCK-trypsin and trypsin inhibitor were dissolved in Buffer 2. The trypsin inhibitor was used at a 2—2.2-fold greater concentration on a weight basis than the TPCK-trypsin.

Materials

Coenzymes, nucleotides, phenylglyoxal and 2,3-butanedione were obtained from Sigma Chemical Company. TPCK-trypsin and soybean trypsin inhibitor were purchased from Worthington Biochemical Corporation.

Results

Steady-state kinetics of energy-independent transhydrogenation

Figs. 1 and 2 show double-reciprocal plots of the velocities of the reactions at varying concentrations of the two substrates 3-acetylpyridine-NAD^+ and NADPH. The analog of NAD^+ is used so that the reaction can be measured spectrophotometrically at a wavelength (375 nm) at which there is minimum interference from the absorbance due to NADPH. When 3-acetylpyridine-NAD^+ was the variable substrate, almost parallel plots were obtained at the lowest fixed concentrations (10 and 24 μM) of the second substrate, NADPH (Fig. 1). The lines became more convergent at higher concentrations of NADPH (97 and 241 μM) intersecting on the left-hand side of the ordinate. The slope of the line decreased to a minimum value at 241 μM NADPH before increasing once again. At the highest concentrations of NADPH (482 and 965 μM) the lines intersected close to the ordinate. Similar, but less dramatic, behaviour was observed when constant fixed concentrations of 3-acetylpyridine-NAD^+ were used with NADPH as the variable substrate (Fig. 2). The convergent nature of the lines obtained at intermediate levels of the fixed substrate suggests that the transhydrogenase reaction follows a sequential (ternary complex) mechanism [21]. At the highest concentrations of the fixed substrate, competition between it and the variable substrate is suggested by the intersection on the ordinate of the lines of the double-reciprocal plot. Segal [22] has discussed this type of kinetic plot in some detail. He suggests that it is indicative of a rapid equilibrium random bireactant system in which the second substrate can also bind at

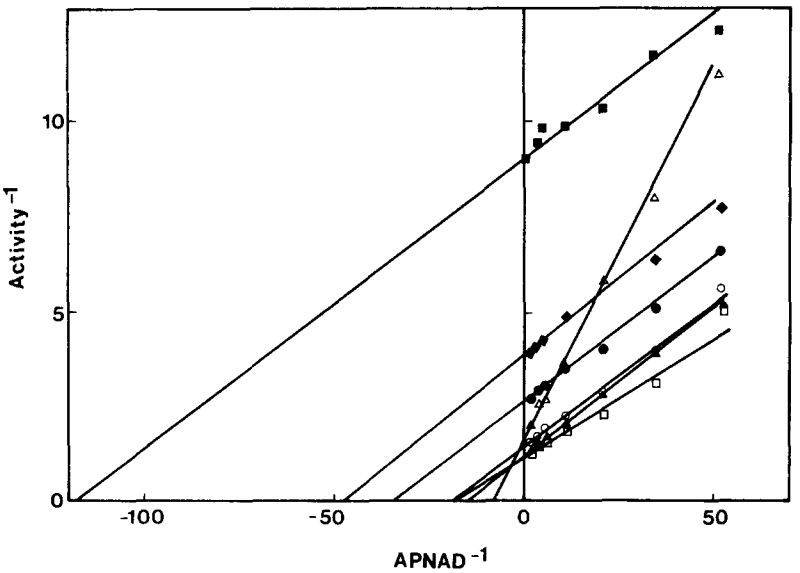

Fig. 1. Effect of the concentration of 3-acetylpyridine-NAD$^+$ (APNAD) on the activity of the energy-independent transhydrogenase at constant concentrations of NADPH (■———■, 10 μM; ◆———◆, 24 μM; ●———●, 48 μM; ○———○, 97 μM; □———□, 241 μM; ▲———▲, 482 μM; △———△, 965 μM). Cell membranes were prepared in Buffer 2. 97.5 μg protein was used in each assay. Activity^{-1} is expressed as (nmol/min per mg protein)$^{-1}$ X 10^3. The concentration of APNAD is expressed as mM.

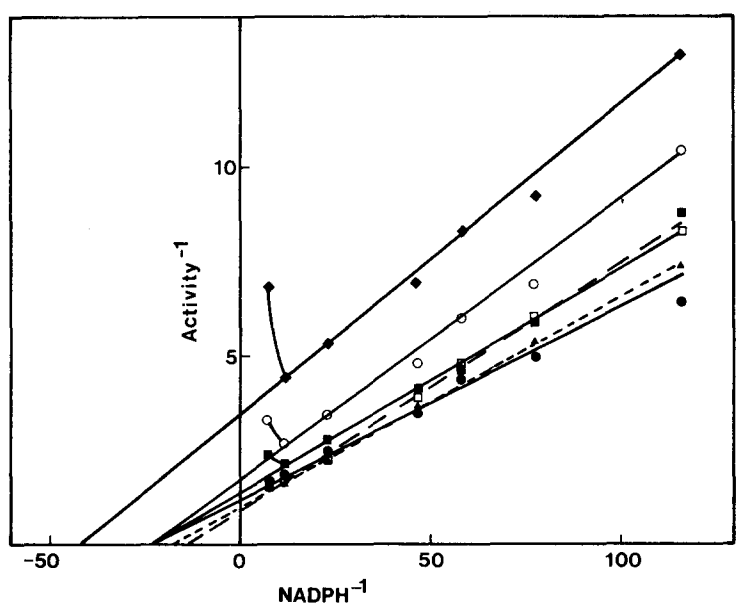

Fig. 2. Effect of the concentration of NADPH on the activity of the energy-independent transhydrogenase at constant concentrations of 3-acetylpyridine-NAD$^+$ (◆———◆, 20 μM; ○———○, 49 μM; ■———■, 98 μM; ●———●, 491 μM; ▲———▲, 982 μM; □———□, 3872 μM). Cell membranes were prepared in Buffer 2. 90 μg protein was used in each assay. Activity^{-1} is expressed as (nmol/min per mg protein)$^{-1}$ X 10^3. The concentration of NADPH is expressed as mM.

the substrate-binding site of the first substrate to form a dead-end complex. At higher concentrations the second substrate will act as a competitive inhibitor of the first substrate. Thus, the results of Figs. 1 and 2 indicate that both 3-acetylpyridine-NAD^+ and NADPH can also bind at the binding site of the other nucleotide. This viewpoint is further supported by the data shown in Fig. 2 where substrate inhibition is observed at higher concentrations of NADPH. The extent of substrate inhibition becomes less as the concentration of 3-acetylpyridine-NAD^+ is increased. These results suggest that substrate inhibition by NADPH is due to binding in the 3-acetylpyridine-NAD^+ site. However, we cannot entirely exclude the possibility that inhibition by the higher concentrations of NADPH is due to the presence of NAD(H), known contaminants of commercially available NADPH.

Most sequential bireactant mechanisms show a rate equation for the velocity of the reaction in the forward direction in the form

$$v = \frac{VAB}{K_s^A K_m^B + K_m^A B + K_m^B A + AB}$$

or

$$v = \frac{VAB}{K_s^B K_m^A + K_m^A B + K_m^B A + AB}$$

where A and B are the concentrations of substrates A and B, respectively, V is the limiting maximum velocity, K_S^A and K_S^B are the dissociation constants of the enzyme-A and enzyme-B complexes, respectively, and K_m^A and K_m^B are the Michaelis constants for A and for B [21,22]. The dissociation and Michaelis constants were evaluated from replots of the data shown in Figs. 1 and 2 [22]. K_S^{NADPH}, K_S^{APNAD}, K_m^{NADPH}, and K_m^{APNAD} were 15, 3.1, 107, and 47 μM, respectively.

The kinetic behaviour demonstrated in Figs. 1 and 2 suggests that the transhydrogenase follows a rapid equilibrium random bireactant mechanism. In this mechanism it is assumed that the substrates 3-acetylpyridine-NAD^+ and NADPH add to, and the products 3-acetylpyridine-NADH and $NADP^+$ are released from, the enzyme in no fixed order. Moreover, the rate-limiting step is the interconversion of the two ternary complexes 3-acetylpyridine-NAD^+ · enzyme · NADPH and 3-acetylpyridine-NADH · enzyme · $NADP^+$. In order to investigate further the kinetic mechanism of the transhydrogenase, product inhibition experiments were carried out. As shown in Table I, 3-acetylpyridine-NADH was competitive with respect to 3-acetylpyridine-NAD^+ but noncompetitive with respect to NADPH whereas $NADP^+$ showed a competitive relationship with NADPH and a noncompetitive relationship with 3-acetylpyridine-NAD^+. This pattern of product inhibition is diagnostic of both rapid equilibrium random and Theorell-Chance bireactant mechanisms [21,22]. Non-rapid equilibrium random and ordered bireactant mechanisms are eliminated since they do not provide this pattern of inhibition by the products of the reaction.

In order to distinguish between the rapid equilibrium random and Theorell-Chance bireactant mechanisms, the kinetics of the transhydrogenase reaction were measured in the presence of alternate substrates. Rudolph and Fromm

TABLE I

INHIBITION BY PRODUCTS OF ENERGY-INDEPENDENT TRANSHYDROGENATION OF 3-ACETYLPYRIDINE-NAD^+ BY NADPH

The experimental conditions were as described in Materials and Methods. K_i and inhibition type were evaluated by Dixon plots [22].

Substrate	Product			
	$NADP^+$		APNADH	
	K_i (μM)	Inhibition type	K_i (μM)	Inhibition type
3-acetylpyridine-NAD^+	440	Non-competitive	38	Competitive
NADPH	110	Competitive	280	Non-competitive

[23] have shown that in rapid equilibrium random bireactant systems the alternate for one substrate will act as a competitive inhibitor with respect to this substrate and as a noncompetitive inhibitor for the second substrate. The inverse relationship is shown when an alternate of the second substrate is used. With Theorell-Chance and ordered bireactant systems the alternate substrate has a competitive relationship with the normal substrate but shows parabolic curves in double-reciprocal plots with respect to the second substrate. As shown in Fig. 3, NAD^+ was a competitive and a noncompetitive inhibitor of 3-acetylpyridine-NAD^+ and NADPH, respectively, with apparent K_i values of 0.16 and 3.2 mM. Deamino-NADPH showed a competitive relationship with NADPH and was a noncompetitive inhibitor with respect to 3-acetylpyridine-NAD^+. The apparent K_i values were 0.48 and 4.2 mM, respectively. These results show that the energy-independent transhydrogenase follows a rapid equilibrium random bireactant mechanism.

The results described above suggest that the transhydrogenase possesses two binding sites. The site at which 3-acetylpyridine-NAD^+, 3-acetylpyridine-NADH, and NAD^+ interact may be described as the 'NAD^+ site'. $NADP^+$, NADPH and deamino-NADPH interact at the 'NADPH site'. The properties of these two sites were explored further by studying the effect of various nucleotides on the transhydrogenase reaction. Dixon plots of the effect of ADP and 5'-AMP on transhydrogenase activity are shown in Fig. 4. ADP and 5'-AMP were competitive inhibitors with respect to 3-acetylpyridine-NAD^+ (K_i, 4.5 and 3.0 mM, respectively) and noncompetitive inhibitors with respect to NADPH (K_i, 18.5 and 21 mM, respectively). In contrast, 2'-AMP showed competitive and noncompetitive relationships with NADPH (K_i, 3.9 mM) and 3-acetylpyridine-NAD^+ (K_i, 25 mM), respectively. These results suggest that 5'-AMP and ADP interact at the NAD^+ site, whereas 2'-AMP is bound to the NADPH site. The lines obtained with 5'-AMP and ADP deviated from linearity at higher concentrations of these nucleotides (Fig. 4). This was particularly noticeable when lower concentrations of either substrate were used. Since high concentrations of K^+ were introduced concomitantly with the neutralized solutions of the nucleotides, the effect on the reaction of KCl at concentrations of K^+ equivalent to that introduced with the nucleotides was examined (Fig. 4). KCl

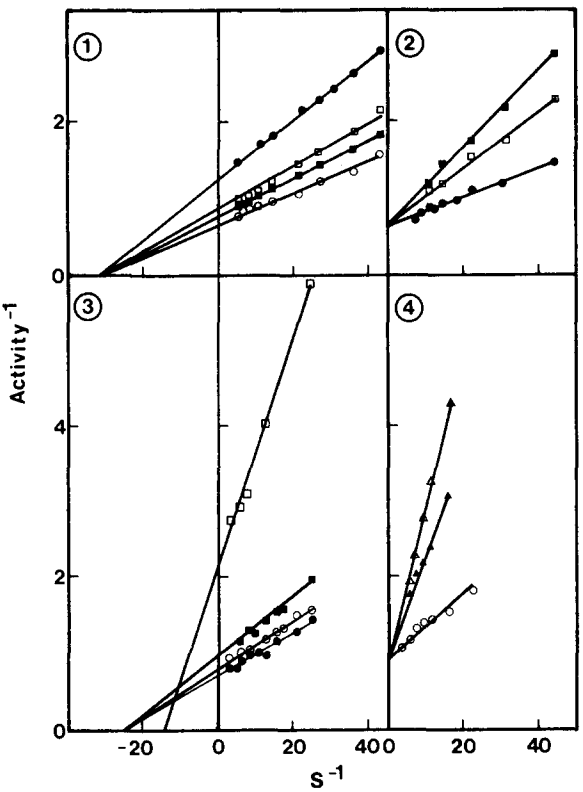

Fig. 3. Inhibition of energy-independent transhydrogenase activity by alternate substrates NAD^+ and deamino-NADPH. In panels 1 and 2 the variable substrate (*S*) is NADPH with 0.9 mM 3-acetylpyridine-NAD^+. In panels 3 and 4 the variable substrate (*S*) is 3-acetylpyridine-NAD^+ with 0.25 mM NADPH. The concentration of the variable substrate is expressed as mM. Activity^{-1} is expressed as (nmol/min per mg protein)$^{-1}$ × 10^3. Panel 1. NAD^+ concentration: ○———○, 0 μM; ■———■, 475 μM; □———□, 855 μM; ●———●, 3440 μM. Panel 2. Deamino-NADPH concentration: ●———●, 0 μM; □———□, 530 μM; ■———■, 796 μM. Panel 3. Deamino-NADPH concentration: ●———●, 0 μM; ○———○, 293 μM; ■———■, 770 μM; □———□, 1290 μM. Panel 4. NAD^+ concentration: ○———○, 0 μM; ▲———▲, 348 μM; △———△, 522 μM. The membranes were prepared in Buffer 2. 52—63 μg protein was used in each assay.

at concentrations up to 100 mM had little effect on the transhydrogenase reaction. Thus, it is possible that the curved plots showing increasing inhibition at higher concentrations of ADP and 5′-AMP are due to the ability of these nucleotides to bind at the NADPH site as well as at the NAD^+ site. As discussed previously, this behaviour was shown also by the two substrates of the reaction.

By analogy with the concept of 'alternate' substrates, NADH may be considered to be an 'alternative' product of the transhydrogenase reaction. It should inhibit the transhydrogenation of 3-acetylpyridine-NAD^+ by NADPH. This effect is shown in Fig. 5 where the results are given as a Dixon plot. Inhibition occurs at concentrations of 0.2—0.4 mM NADH depending on the initial concentrations of the reactants. Stimulation of the reaction is observed at lower concentrations of NADH. This is not due to the reduction of 3-acetylpyridine-NAD^+ by NADH since this reaction occurs at less than 1% of the rate with NADPH. Fig. 6 shows the effect of the concentration of 3-acetylpyridine-

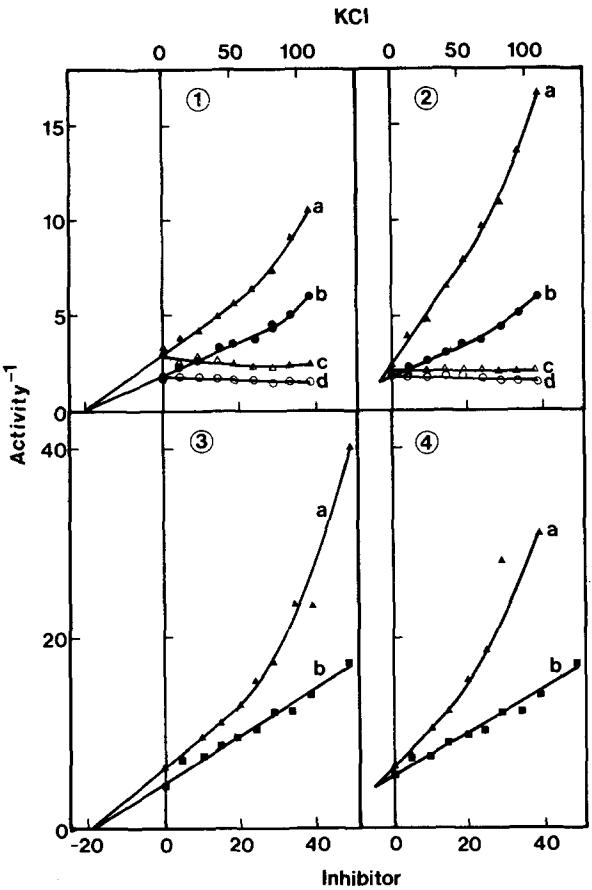

Fig. 4. Effect of substrate analogs and KCl on energy-independent transhydrogenase activity. In panels 1 and 2 the inhibitor is 5'-AMP (curves a, b) or KCl (curves c, d). The inhibitor in panels 3 and 4 is ADP. Curves b and d in panels 1 and 2 were run under 'standard' assay conditions (466 μM NADPH; 963 μM 3-acetylpyridine-NAD$^+$). For the other curves the standard assay was modified as follows. Panel 1, curves a and c: NADPH, 57 μM. Panel 2, curves a and c, 3-acetylpyridine-NAD$^+$, 241 μM. Curves b in panels 3 and 4 were run under 'standard' assay conditions (558 μM NADPH; 1060 μM, 3-acetylpyridine-NAD$^+$). The concentrations of NADPH and 3-acetylpyridine-NAD$^+$ in curves a were 112 μM (panel 3) and 353 μM (panel 4), respectively. The membranes were prepared in Buffer 2 and used at 105 μg (panels 1 and 2) or 275 μg (panels 3 and 4) protein per assay. The concentration of inhibitors and of KCl is expressed as mM. Activity^{-1} is given as (nmol/min per mg protein)$^{-1}$ $\times$ 10^3.

NAD$^+$ and of NADPH on the extent of stimulation by a fixed concentration (0.104 mM) of NADH. Both the maximum velocity of the reaction and the apparent K_m of the reaction were affected. The K_m values in the presence of this concentration of NADH were 31 μM and 192 μM for NADPH and 3-acetylpyridine-NAD$^+$, respectively. These results suggest that there may be an allosteric site for NADH on the transhydrogenase. NADH binds to this site at low concentrations to induce a conformational change in the enzyme which results in increased activity. Higher concentrations of NADH cause inhibition presumably by competition with 3-acetylpyridine-NAD$^+$ for binding at the NAD$^+$ site. Further evidence for a conformational change induced by low concentrations of NADH will be given in the next section.

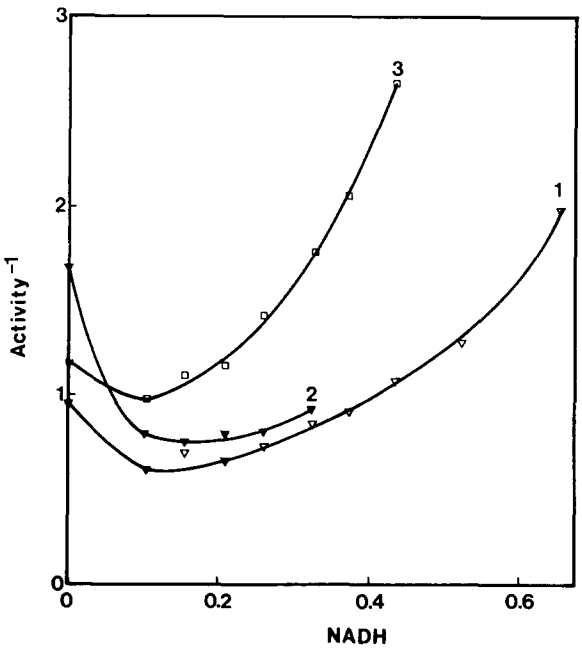

Fig. 5. Effect of NADH on energy-independent transhydrogenase activity. Curve 1: 1.08 mM 3-acetylpyridine-NAD$^+$ (APNAD), 0.58 mM, NADPH. Curve 2: 1.08 mM APNAD, 0.116 mM NADPH. Curve 3: 0.358 mM APNAD, 0.58 mM NADPH. The membranes were prepared in Buffer 3 and used at 50 μg protein per assay. The concentration of NADH is expressed as mM. Activity^{-1} is given as (nmol/min per mg protein)$^{-1}$ $\times$ 10^3.

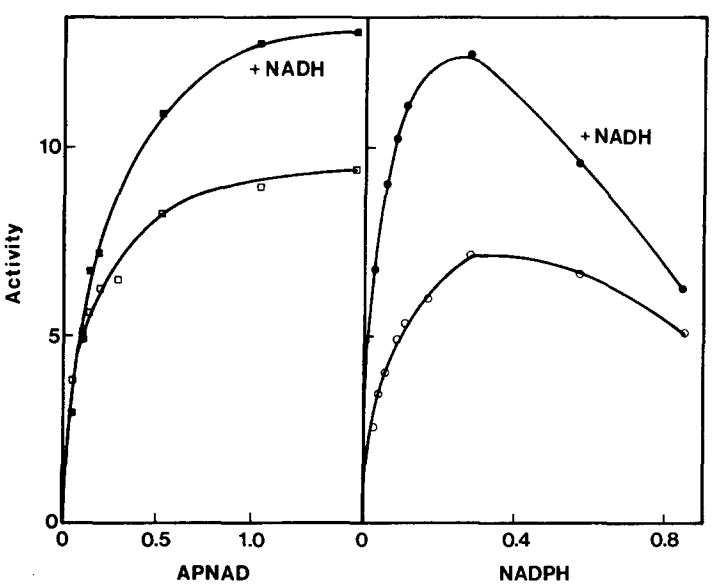

Fig. 6. Effect of NADH on energy-independent transhydrogenase activity at various concentration of substrate. Substrate concentration is expressed as mM. Activity is given as (nmol/min per mg protein)$^{-1}$ $\times$ 10^{-2}. The concentrations of NADPH (left panel) and 3-acetylpyridine-NAD$^+$ (APNAD) (right panel) were 0.5 and 1.0 mM, respectively. NADH, where present (solid points), was at a concentration of 104 μM. Membranes were prepared in Buffer 3 and used at 50 μg per assay.

Reaction of the energy-independent transhydrogenase with 2,3-butanedione and phenylglyoxal

2,3-Butanedione in borate buffer and phenylglyoxal have been employed to specifically modify arginyl residues in proteins and to identify the presence of arginyl residues in the nucleotide-binding sites of enzymes [24—26]. The guanidino group of the arginyl residue reacts with 2,3-butanedione to form a 4,5-dimethyl-4,5-dihydroxy-2-imidazoline complex. Borate reacts with this *cis*-diol to stabilize the complex. However, 2,3-butanedione has also been observed to form a stable complex with arginyl residues in the absence of borate [27].

The effect of phenylglyoxal and 2,3-butanedione in borate buffer on transhydrogenase activity is shown in Fig. 7. Enzyme activity was inhibited by both compounds. The inactivation rates obeyed pseudo-first-order kinetics with respect to enzyme activity. The pseudo-first-order rate constants were calculated from the slopes of the lines shown in Fig. 7. The reaction order with respect to each inhibition was determined from a plot of the logarithm of the pseudo-first-order rate constant versus the logarithm of the concentration of the inhibitor (Fig. 7, insets). In this type of plot a straight line should be obtained with a slope equal to n, where n is the number of molecules of inhibitor reacting with each active site of the enzyme to produce an inactive enzyme-inhibitor complex. The value of n for 2,3-butanedione and phenylglyoxal were 0.8 and 1.1, respectively. This suggests that inactivation was due to the modification of one arginyl residue per active site of the transhydrogenase.

The reaction of the transhydrogenase with 2,3-butanedione in borate buffer was explored in more detail. If the modifiable arginyl residues are involved in the binding of the nucleotide coenzymes, as seems likely from the results with other enzymes [25,26], then it might be expected that the presence of substrates in the 2,3-butanedione reaction mixture would prevent binding of this compound and the accompanying inhibition. The results of such an experiment are shown in Fig. 8. The substrates NAD^+ and $NADP^+$, and the site-specific inhibitors 5′-AMP and 2′-AMP, when present during the incubation of the membranes with 2,3-butanedione diminished the extent of inhibition of transhydrogenase activity. As shown in Table II, the pseudo-first-order rate constant for the inhibition reaction was much lower for a mixture of $NADP^+$ and NAD^+ than when each nucleotide coenzyme was used alone. These results suggest that the inhibitor is reacting with arginyl residues in the vicinity of the substrate-binding sites, and that binding of substrate at both NAD^+ and NADPH binding sites affords greater protection, possibly because of cooperative or steric interaction between the sites.

As described in a previous section, low concentrations of NADH were found to stimulate the transhydrogenation of 3-acetylpyridine-NAD^+ by NADPH. This was attributed to a conformational change at the active site induced by the binding of NADH. Further support for this hypothesis was obtained from experiments in which the effect of NADH and NADPH on the inhibition of transhydrogenase activity by 2,3-butanedione was measured. Low concentrations of both NADH and NADPH enhanced the rate of inhibition of enzyme activity by 2,3-butanedione (Fig. 9). NADH was more effective than NADPH. The enhancement by the reduced pyridine nucleotide coenzymes was reversed at higher concentrations where protection against inhibition was observed.

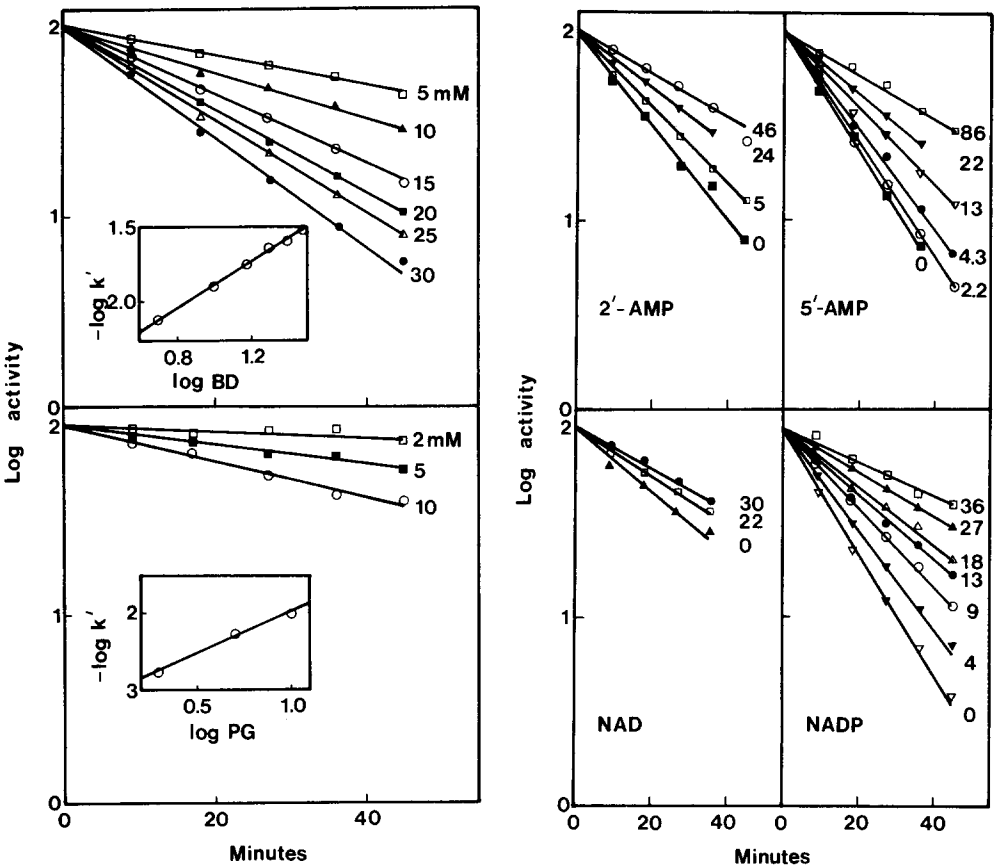

Fig. 7. Kinetics of inactivation of the energy-independent transhydrogenase by 2,3-butanedione (BD) (upper panel) and phenylglyoxal (PG) (lower panel). The membranes were prepared in Buffer 3 and incubated at 22°C at a concentration of 1.1 mg and 1.96 protein/ml with the indicated concentrations of 2,3-butanedione and phenylglyoxal, respectively. Samples were withdrawn and assayed at timed intervals. Activity is expressed as a percentage of the control activity taken at the onset of incubation. The insets show the relationship between the pseudo-first-order rate constant of inactivation (k^1) and the inhibitor concentration (expressed as mM) · k^1 is expressed as min^{-1}.

Fig. 8. Effect of substrates and substrate analogs on the inactivation of the energy-independent transhydrogenase by 2,3-butanedione. Membranes at a concentration of 1.0 (top panels) and 1.4 (bottom panels) mg protein/ml Buffer 3 were incubated at 22°C with 53.7 mM 2,3-butanedione, in the absence or presence of the indicated millimolar concentrations of substrates and substrate analogs. Samples were withdrawn at timed intervals for assay. Activity is expressed as a percentage of the control activity taken at the onset of incubation.

Thus, complete protection was afforded by 17 mM NADH. Enhancement of 2,3-butanedione inhibition was not produced by comparable concentrations of the oxidized coenzymes. Depending on the concentration used, these compounds were either ineffective or protected the enzyme against modification (Fig. 8).

Hoek et al. [10] have shown that the transhydrogenases of *E. coli* and submitochondrial particles are extremely sensitive to trypsin treatment. The effect of trypsin treatment is shown in Fig. 10. Above a certain concentration,

TABLE II

EFFECT OF SUBSTRATES ON INHIBITION OF ENERGY-INDEPENDENT TRANSHYDROGENASE BY 2,3-BUTANEDIONE

Membranes at a concentration of 1.84 mg protein/ml in Buffer 3 were incubated at 22°C with 53.7 mM 2,3-butanedione in the presence or absence of the indicated concentrations of substrates. Samples were removed at timed intervals for assay of transhydrogenase activity. Pseudo-first order rate constants for the inactivation of the enzyme are expressed as min^{-1}.

Addition	Concentration (mM)	Pseudo-first-order rate constant ($\times 10^3$)
None	—	13.5
NAD^+	31	6.9
$NADP^+$	28	8.8
NAD^+	15.5	3.7
plus $NADP^+$	14	

which depends on the particular coenzyme, protection against inactivation of the transhydrogenase by trypsin is afforded by the presence of $NADP^+$, NADH or NAD^+. At lower concentrations, NADPH and NADH can increase the rate of inhibition by trypsin. This effect is not shown by the oxidized coenzymes. (The enhancement produced by 1.3 mM $NADP^+$ in Fig. 10 is not considered to be of significance on the basis of the results of other experiments.) The increase in the rate of trypsin digestion on binding of the reduced coenzymes suggests that these compounds can induce a conformational change in the enzyme.

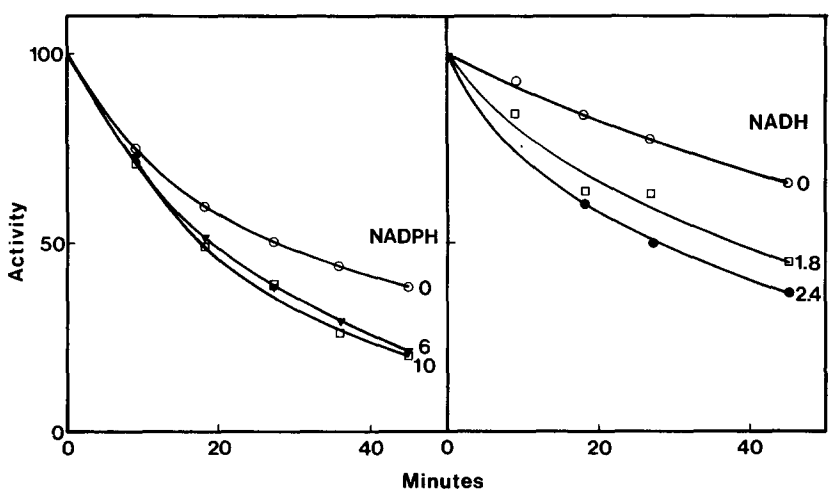

Fig. 9. Effect of NADPH and NADH on the inhibition of the energy-independent transhydrogenase by 2,3-butanedione. Membranes at a concentration of 2.3 (left panel) and 1.74 (right panel) mg protein/ml Buffer 3 were incubated at 22°C with 53.7 mM 2,3-butanedione in the presence and absence of the indicated millimolar concentrations of NADPH or NADH. Samples were withdrawn and assayed at timed intervals. Activity is expressed as a percentage of the control activity at the onset of incubation.

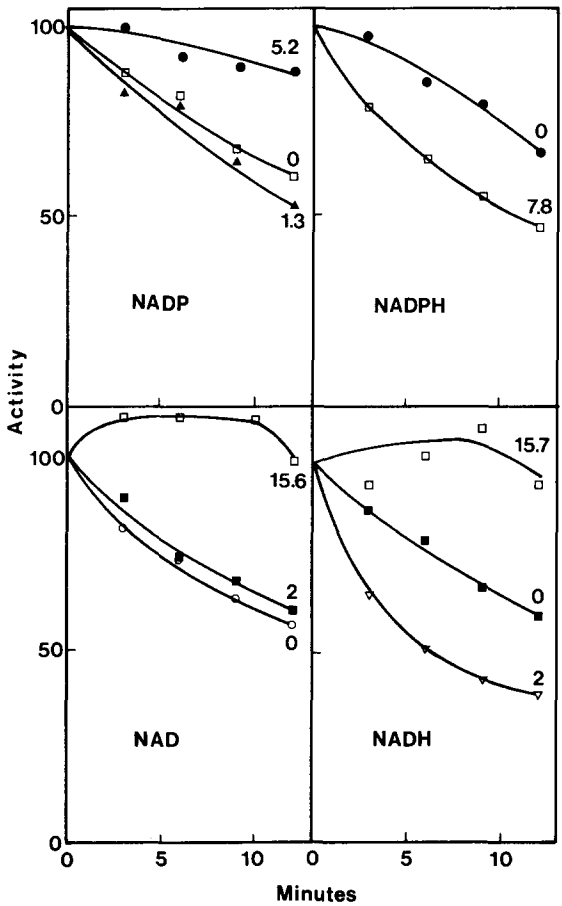

Fig. 10. Effect of substrates on the inactivation of the energy-independent transhydrogenase by TPCK-trypsin. Membranes in Buffer 2 were incubated at 22°C at a TPCK-trypsin:protein ratio (μg/mg) of 0.9, 0.42, 0.84, and 0.42 for the experiments using NADP, NADPH, NAD and NADH, respectively. Samples were withdrawn for assay and digestion stopped by addition of trypsin inhibitor. The samples were kept at 0°C and assayed at the end of each experiment. Activity is expressed as a percentage of the control activity at the onset of incubation. The concentration of substrate is expressed as mM.

Discussion

The results described in this paper indicate that the energy-independent transhydrogenase of *E. coli* follows a rapid equilibrium random bireactant mechanism. Our conclusion is based on the following evidence: (a) the reciprocal velocity-substrate concentration plots are typical of a rapid equilibrium random bireactant mechanism in which each substrate can also interact with the binding site of the other substrate [22]; (b) the type of inhibition produced by the products of the reaction, $NADP^+$ and 3-acetylpyridine-NADH are diagnostic of rapid equilibrium random bireactant or Theorell-Chance mechanisms [22,28]; (c) the type of inhibition produced by the presence of an alternate substrate in the reaction mixture is typical of rapid equilibrium random or ping-pong bireactant mechanisms [23]. The ping-pong bireactant

mechanism is eliminated by the convergent nature of the reciprocal velocity-substrate concentration plots. These results are in accord with the conclusions of Hanson [9] for a detergent-solubilized preparation of the transhydrogenase from the membranes of *E. coli*, although there are some differences in the magnitude of the Michaelis and inhibitor constants derived in the two studies which may be attributable to the use of membrane-bound and solubilized forms of the enzyme, respectively. In contrast, Houghton et al. [8] concluded that the membrane-bound enzyme of *E. coli* followed a Theorell-Chance mechanism. However, their data are compatible with the conclusions of Hanson [9] and ourselves. Following the original paper of Cleland [28] they had assumed that the pattern of product inhibition observed in their experiments could distinguish between Theorell-Chance and rapid equilibrium random bireactant mechanisms. This was subsequently shown to be incorrect [29].

The experiments with 2,3-butanedione and phenylglyoxal suggest that arginyl residues are involved at the active site of the transhydrogenase. Confirmation of the identity of the modified residues is not possible at present since the transhydrogenase, although solubilized [9,16,30] has not been purified to homogeneity. Experiments with other enzymes indicate that the 2,3-butanedione or phenylglyoxal-modifiable residues are probably involved in the binding of nucleotide substrates at the active site [25,26]. Our results showing protection against inhibition by the substrates of the transhydrogenase are consistent with this hypothesis. The substrate analogs 5′-AMP and 2′-AMP were shown to interact at the NAD^+ and $NADP^+$-binding sites of the transhydrogenase. The presence of these nucleotides during reaction of the enzyme with 2,3-butanedione afforded protection against inactivation by this inhibitor. The concentrations of these nucleotides and of the substrates required to give protection are high. It is possible to calculate a dissociation constant, K_D, for the enzyme-protective agent complexes using the method of Schuber et al. [31]. These workers have shown that

$$\frac{k}{k^0} = \frac{K_D}{K_D + P}$$

where k^0 and k are the pseudo-first order rate constants for the reaction of butanedione with the enzyme in the absence and presence of the protective agent, and P is the concentration of the protective agent. Using this equation, we have calculated dissociation constants of about 23 and 38 mM for 5′-AMP and 2′-AMP. The K_i values for the inhibition of transhydrogenase activity by 5′-AMP and 2′-AMP are 3.0 and 3.9 mM, for binding at the NAD^+ and $NADP^+$ sites, respectively. The reason for the approximately 10-fold difference between the two sets of values is not clear at present. However, one possible factor may be that binding of substrate at both sites is required for complete protection of the modifiable arginyl residue(s). Thus, a combination of NAD^+ and $NADP^+$ was found to give more effective protection than when either nucleotide coenzyme was used alone. A similar effect has been observed with the transhydrogenase of beef-heart submitochondrial particles [11] and *Rhodospirillum rubrum* chromatophores [32].

Fisher and his coworkers have supplied evidence for substrate-induced conformational changes in the transhydrogenase of submitochondrial particles

[12—15]. They have suggested that the enzyme exists in at least three different conformations: the native enzyme, the $NADP^+$-enzyme complex, and the NADPH-enzyme complex. This hypothesis is based on the following results. Transhydrogenase activity was stabilized to thermal inactivation by NADPH and labilized by $NADP^+$. Tryptic inactivation of the native enzyme or inhibition by *N*-ethylmaleimide was enhanced in the presence of NADPH while $NADP^+$ had little effect on tryptic inactivation but afforded protection against the sulfhydryl modification by *N*-ethylmaleimide. NAD^+ and NADH had no effect on *N*-ethylmaleimide inactivation or on the thermostability of the enzyme. These coenzymes protected against proteolytic digestion of beef heart but not of rat liver submitochondrial particles. Houghton et al. [16] have examined the effect of *N*-ethylmaleimide on the energy-independent transhydrogenase of *E. coli* membranes. Their results resembled those of Fisher and his coworkers with the beef-heart submitochondrial system in that NADPH enhanced the rate of inhibition by the sulfhydryl inhibitor whereas $NADP^+$ afforded protection. In contrast to the mitochondrial enzyme, NAD^+ was also able to protect against inhibition by *N*-ethylmaleimide.

The experiments described in this paper also indicate the presence of different conformational forms of the transhydrogenase. However, the results with our system differ in detail from those reported by Fisher and his coworkers for the mitochondrial enzyme. Both NADH and NADPH at low concentrations enhanced the rate of tryptic digestion and the inhibition by 2,3-butanedione. These coenzymes afforded protection at higher concentrations. In contrast, NAD^+ and $NADP^+$ did not significantly enhance either tryptic digestion or modification of the arginyl residues but protected against inhibition of the enzyme by these agents. These results, together with those of Houghton et al. [16], suggest that there is a conformation of the transhydrogenase in the *E. coli* membrane in which certain arginyl and sulfhydryl residues are only partially accessible to modifying agents. However, in the presence of low concentrations of reduced pyridine nucleotide coenzymes the enzyme alters it conformation such that these residues become more accessible. It is not clear if the conformational change is induced by the presence of the reduced coenzymes at the active site, in which case the conformational change might occur as part of the catalytic cycle, or if the reduced coenzymes bind at an allosteric site. We favour the latter possibility, since addition of low concentrations of NADH (it was not possible to test NADPH) to the enzyme stimulated its activity in the transhydrogenation of 3-acetylpyridine-NAD^+ by NADPH. The finding of these coenzyme-binding sites on the transhydrogenase of *R. rubrum* chromatophores is consistent with another coenzyme-binding site in addition to those at the active site [32].

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