The Effect of Cyclic 3′,5′-Adenosine Monophosphate on Yeast Glyceraldehyde-3-phosphate Dehydrogenase. II. Initial Velocity Kinetic Studies†

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ABSTRACT: Glyceraldehyde-3-phosphate dehydrogenase from yeast exhibits a unique kinetic behavior in the presence of cyclic 3′,5′-AMP in initial velocity kinetic studies. The enzyme is inhibited noncompetitively by cyclic 3′,5′-AMP with respect to both NAD^+ and glyceraldehyde 3-phosphate indicating that cAMP binds to a site other than the catalytic site, in agreement with direct binding studies. In the absence of cyclic 3′,5′-AMP, velocity-NAD^+ curves exhibit an intermediary plateau region, corresponding to the activity of the first two subunits, while velocity-glyceraldehyde 3-phosphate curves exhibit normal Michaelis-Menten kinetics. In the presence of cyclic 3′,5′-AMP, the shape of the velocity-G3P curves is unaltered, but at least two additional plateau regions are observed in velocity-NAD^+ curves. It is suggested that the binding of cyclic 3′,5′-AMP causes a conformational change in individual subunits resulting in increased negative cooperative interactions between the NAD^+-binding sites, consistent with a ligand induced sequential model. It is also suggested that a more gross conformational change occurs after 50% saturation of the enzyme by NAD^+, indicating that the enzyme may exhibit half-site reactivity.

Glyceraldehyde-3-phosphate dehydrogenase from yeast has been shown to be strongly inhibited by cyclic 3′,5′-AMP (cAMP)[1] and inhibited to a lesser extent by other adenine-containing compounds (Yang and Deal, 1969). In these studies, the enzyme was shown to exhibit normal Michaelis-Menten kinetics with NAD^+ as the variable substrate. The instantaneous inhibition of enzyme activity by adenine-containing compounds was suggested to be due to their competition with NAD^+ for the NAD^+-binding site on the enzyme.

Initial velocity kinetic studies by Koshland *et al.* (1970), however, indicated more complex behavior in velocity-NAD^+ curves. Specifically, intermediary plateau regions were observed in velocity-NAD^+ saturation curves at nonsaturating concentrations of G3P. At all concentrations of G3P, Hill plots of NAD^+ were biphasic, with a break occurring at approximately 50% of the maximum velocity. In contrast, when glyceraldehyde 3-phosphate was used as the variable substrate at several fixed concentrations of NAD^+, normal Michaelis-Menten kinetics were observed (Koshland *et al.*, 1970). This unusual kinetic behavior was consistent with physicochemical studies on NAD^+ binding to the enzyme and resulted in the suggestion that NAD^+ caused both poitive and negative cooperative behavior in yeast GPD (Cook and Koshland, 1970).

Results presented in the preceding paper (Milne and Cook, 1974) indicate that cAMP may not be a simple competitive inhibitor of the enzyme, but rather, an allosteric effector. It was therefore of interest to examine the effect of cAMP on the kinetic properties of the yeast enzyme. The results of such studies are presented in this paper and indicate a unique, complex response of this enzyme to cAMP concentration.

Experimental Section

Materials. Glyceraldehyde-3-phosphate dehydrogenase was purified from Red Star brand baker's yeast following the procedure of Krebs (1955). After four crystallizations, the enzyme exhibited an OD 280:260 ratio of 1.5-1.6. The enzyme appeared to be homogeneous when tested by cellogel electrophoresis in Tris-borate buffer (pH 8.6) (Boyer *et al.*, 1963) and by disc gel electrophoresis (Ornstein and Davis, 1964). Prior to initial velocity kinetic studies, crystals of the enzyme were centrifuged down, dissolved in, and dialyzed against 0.05 M sodium pyrophosphate buffer (pH 8.5), containing 1.0 mM EDTA. In all experiments reported here, an enzyme preparation with a specific activity of 200,000-220,000 was used. One unit is defined as an increase of 0.001 at 340 mμ per min per mg of protein. The enzyme was found to be stable for no longer than 3 months when stored as the crystalline suspension in ammonium sulfate containing EDTA (1 mM) and dithiothreitol (1mM) at 0-4°.

Assay Procedure. Enzyme assays and kinetic measurements were performed at 24-25° with a Gilford recording spectrophotometer, Model 2400, equipped with dual thermoplates. The temperature was maintained at 24-25° with a Haake thermostat. The enzyme was checked periodically for any denaturation during experiments by using a standard reaction mixture which contained 50 μmol of sodium pyrophosphate (pH 8.5), 10 μmol of sodium arsenate, 0.468 μmol of glyceraldehyde 3-phosphate, and 0.650 μmol of NAD^+ in 1.2 ml total volume.

Due to the complexity of the kinetic results to be presented in the Results section, it is necessary to describe our assay procedure fully. Since 30-40 experimental points were routinely obtained for each velocity-substrate curve, it was convenient to set up eight cuvets in advance in an increasing concentration series. The second concentration series overlapped two points of the first series, etc. After completion of the total series, several random concentrations were attempted to complete the total curve. Temperature equilibrium was attained by allowing the cuvets to remain in the thermostated cuvet holder until equilibration had occurred (approximately 5 min). The reac-

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[1] Abbreviations used are: GPD, glyceraldehyde-3-phosphate dehydrogenase; cAMP, cyclic 3′,5′-adenosine monophosphate; G3P, glyceraldehyde 3-phosphate; n_H, Hill coefficient.

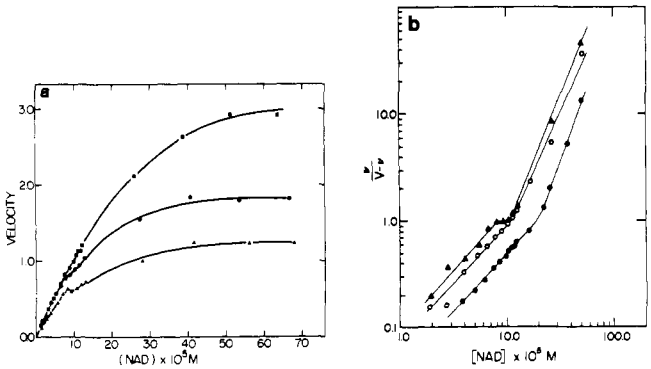

FIGURE 1: The effect of NAD^+ concentration on the initial velocity of glyceraldehyde 3-phosphate oxidation catalyzed by yeast glyceraldehyde-3-phosphate dehydrogenase. Conditions: 0.05 M sodium pyrophosphate buffer, containing 1 mM EDTA (pH 8.5), 25°. Velocity is expressed as the increase in absorbance at 340 mμ/min. (▲) 0.31 mM G3P; (●) 0.62 mM G3P; (■) 1.24 mM G3P.

tion was initiated, routinely, by the addition of enzyme. Control experiments indicated that identical results were attained when the reaction was initiated by NAD^+ or glyceraldehyde 3-phosphate. After addition of the last component and mixing, the reaction rate was found to be linear for 30 sec. Under these conditions, individual assays are quite reproducible.

In the results presented in this paper, approximately 40% of the limiting substrate, NAD^+ was consumed at the lowest NAD^+ concentrations attempted. At the highest NAD^+ concentration, less than 5% of the total NAD^+ was consumed. Less than 1% of the total glyceraldehyde 3-phosphate concentration was consumed in any of the velocity-NAD^+ curves to be presented. It therefore appears unlikely that any spontaneous hydrolysis of G3P at pH 8.5 would have an effect on the shape of the curves presented.

The yeast enzyme used throughout this study was not subjected to charcoal treatment. The enzyme, as isolated, exhibited an OD 280:260 ratio of 1.5, indicating 1.5 mol of bound NAD^+ by the method of Fox and Dandliker (1956) or Kirschner *et al.* (1971). Heat precipitation followed by a fluorometric determination of the released NAD^+ (Lowry *et al.*, 1957) indicated less than 0.05 mol of NAD^+ bound/mol of enzyme. The nature of the bound material has not yet been further determined. Charcoal treatment of the enzyme by the method of Murdock and Koeppe (1964) resulted in an enzyme with an OD 280:260 ratio of 2.1. Control experiments indicated that charcoal treatment of the enzyme did not alter the kinetic results presented in this paper.

NAD^+ was purchased from Boehringer und Sohne, Mannheim. Glyceraldehyde 3-phosphate diethylacetal barium salt was purchased from Sigma and converted to the free acid as described by Sigma. Cyclic 3′,5′-AMP, sodium salt, was purchased from Sigma Chemical Co.

Results

Initial Velocity Studies in the Absence of cAMP. In an earlier study (Koshland *et al.*, 1970), it was shown by steady-state kinetic treatment that when NAD^+ was used as the variable substrate at several fixed concentrations of glyceraldehyde 3-phosphate at 25°, several deviations from Michaelis–Menten behavior occurred. Specifically, a short intermediary plateau region was observed in the region of $8\text{–}12 \times 10^{-5}$ M NAD^+ at nonsaturating concentrations of G3P. At saturating glyceraldehyde 3-phosphate concentrations, the intermediary plateau region was not observed within experimental error. Varying degrees of sigmoidal or cooperative behavior was observed in repeat experiments.

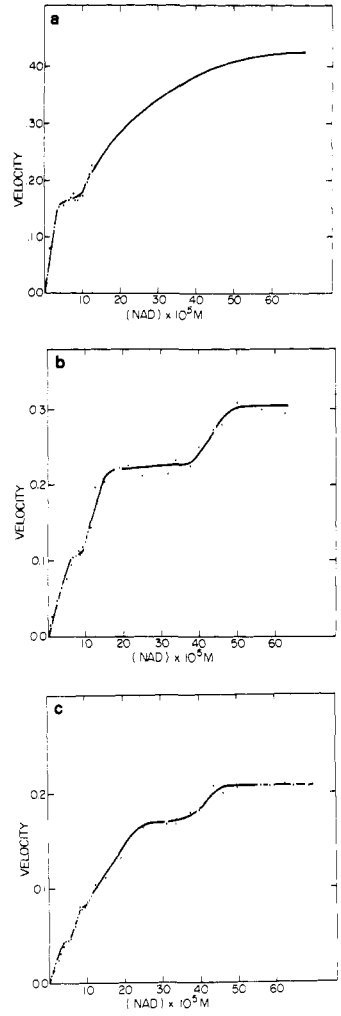

FIGURE 2: The effect of cyclic 3′,5′-AMP on initial velocity kinetic studies with varying NAD^+ concentration and a fixed G3P concentration (0.77 mM). Conditions: 0.05 M sodium pyrophosphate buffer, containing 1 mM EDTA (pH 8.5), 25°. Velocity is expressed as the increase in absorbance at 340 mμ/min. (a) no cAMP; (b) 0.150 mM cAMP; (c) 0.300 mM cAMP.

In the present study, when NAD^+ was used as the variable substrate at several fixed concentrations of G3P at 25° (Figure 1a), essentially identical results were obtained, except that no obvious cooperative behavior was observed at low NAD^+ concentrations. An intermediary plateau region was observed at approximately $7\text{–}12 \times 10^{-5}$ M NAD^+, with nonsaturating concentrations of G3P. Plotting the data of Figure 1a in a Hill plot gives n_H values of approximately 1 below 50% saturation and approximately 2.5 above 50% saturation at *all* G3P concentrations (Figure 1b). The biphasic nature of the Hill plots with a change in slope at approximately 50% saturation, *i.e.*, when the velocity was half the maximum velocity, is analogous to the results previously observed (Koshland *et al.*, 1970). The n_H values are significantly different, however, reflecting the lack of cooperative behavior at low NAD^+ concentrations.

Initial Velocity Studies in the Presence of cAMP. Cyclic-3′,5′-AMP has been shown to be a potent inhibitor of glyceraldehyde-3-phosphate dehydrogenase (Yang and Deal, 1969). This observation provided an opportunity to examine the effect of an inhibitor on an enzyme system which exhibited intermediary plateau regions. A fine detailed kinetic study was therefore undertaken to determine the effect of cAMP on the kinetic

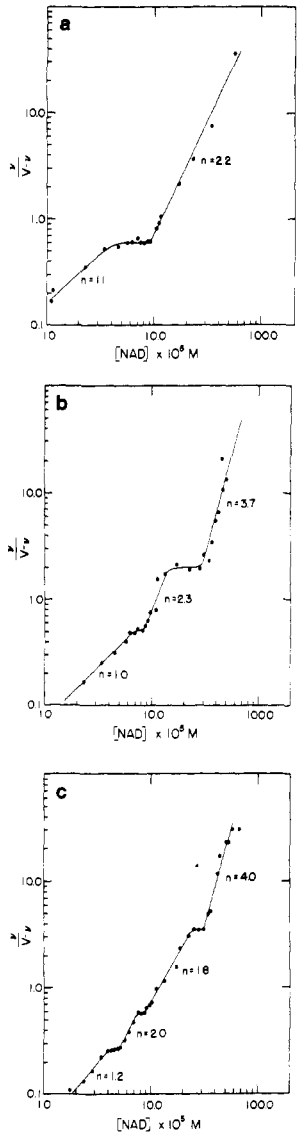

FIGURE 3: Hill plots of the data from Figure 2. Conditions as described in the legend of Figure 2. The lines have been fitted by eye. (a) Data from Figure 2a (no cAMP); (b) data from Figure 2b (0.15 mM cAMP); (c) data from Figure 2c (0.30 mM cAMP).

patterns exhibited by the yeast enzyme. Routinely 30–40 experimental points were obtained for each rate-concentration curve.

The effect of increasing cAMP concentrations on the activity of the enzyme, when NAD^+ was the variable substrate and G3P was fixed, is shown in Figure 2. Glyceraldehyde 3-phosphate concentration was fixed at 0.773 mM, a nonsaturating level. In the absence of cAMP (Figure 2a), the rate-concentration curve was analogous to Figure 1 at nonsaturating levels of G3P. The intermediary plateau region occurred at $4\text{–}10 \times 10^{-5}$ M NAD^+. The plateau region is more pronounced due to the greater number of experimental points obtained. In the presence of low concentrations of cAMP (0.15 mM), approximately 25% inhibition occurred, but a second intermediary plateau region was observed at $15\text{–}35 \times 10^{-5}$ M NAD^+ (Figure 2b). As the cAMP concentration was increased to 0.30 mM, a third small plateau region appeared at lower NAD^+ concentrations ($3\text{–}5 \times 10^{-5}$ M NAD^+) (Figure 2c). The maximum velocity was inhibited approximately 50%. At cAMP concentrations above 0.6 mM (approximately 70% inhibition), no intermediary plateau regions could be discerned due to low velocities and consequent high experimental error. In the absence of cAMP, the double reciprocal plots are curved and biphasic with two apparent V_{max} values of 0.16 and 0.48, respectively. In the most complex curve obtained in the presence of 0.30 mM cAMP, at least four apparent V_{max} values can be estimated. A double reciprocal plot of the total data in Figure 2 indicated that the inhibition by cAMP was noncompetitive with respect to NAD^+.

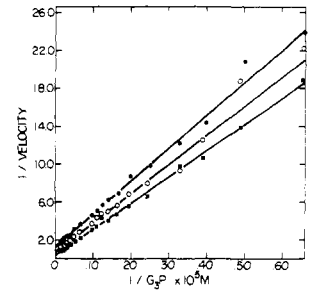

FIGURE 4: The effect of cyclic 3′,5′-AMP on initial velocity kinetic studies with varying G3P concentration and a fixed NAD^+ concentration (0.625 mM), presented in the double reciprocal form. Conditions: 0.05 M sodium pyrophosphate buffer, containing 1 mM EDTA (pH 8.5), 25°. Velocity is expressed as the increase in absorbance at 340 mμ/min. (■) no cAMP; (○) 0.28 mM cAMP; (●) 0.56 mM cAMP.

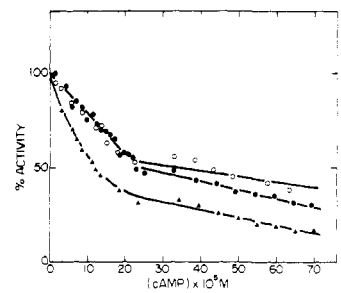

FIGURE 5: The effect of increasing cyclic 3′,5′-AMP concentrations on initial velocity kinetic studies at a fixed G3P concentration (0.87 mM) and three different NAD^+ concentrations. Conditions: 0.05 M sodium pyrophosphate buffer, containing 1 mM EDTA (pH 8.5), 25°. (○) 0.63 mM NAD^+; (●) 0.324 mM NAD^+; (▲) 0.125 mM NAD^+.

Plotting the data of Figure 2 in the Hill plot gives increasingly more complex patterns as cAMP concentration is increased (Figure 3). In the absence of cAMP, two n_H values of 1.1 and 2.2 are observed (Figure 3a). In the presence of 0.15 mM cAMP, three n_H values of 1.0, 2.3, and 3.7 are observed (Figure 3b), while 0.30 mM cAMP, four n_H values of 1.2, 2.0, 1.8, and 4.0 are observed (Figure 3c).

When G3P is used as the variable substrate and NAD^+ is held constant, normal Michaelis-Menten behavior has been observed (Koshland *et al.*, 1970). This conclusion was confirmed in the present study. In the presence of increasing concentrations of cAMP, enzyme activity was inhibited but no change in the shape of the rate-concentration curves was observed. When the data are plotted in a double reciprocal plot, linear noncompetitive inhibition by cAMP was observed with respect to G3P (Figure 4).

The inhibition by cAMP was examined in detail by measuring the velocity of the reaction as a function of cAMP concentration at a fixed nonsaturating concentration of G3P (0.869 mM), and three different fixed concentrations of NAD^+ (0.122, 0.324, and 0.636 mM) (Figure 5). The inhibition curves in all cases appear to be biphasic. When NAD^+ was saturating or near saturating, 50% inhibition occurred at approximately 0.22 mM cAMP. When NAD^+ was fixed at a lower concentra-

tion, 50% inhibition occurred at approximately 0.12 mM cAMP. When the data of Figure 5 are plotted as the logarithm of per cent activity vs. cAMP concentrations, analogous biphasic curves are obtained.

Other nucleotides have also been examined to determine the specificity of the cAMP effect. The following nucleotides have been shown to have no inhibitory effect when tested at a concentration of 0.2 mM: ATP, ADP, AMP, CP, GTP, cyclic 3′,5′-GMP, and cyclic 3′,5′-IMP.

Discussion

The most striking features of the kinetic curves of the yeast enzyme are their deviations from conventional Michaelis–Menten behavior. Before attempting to explain this behavior, it is necessary to discuss the validity of the experimental data presented. The obvious question we have asked ourselves is whether the plateau regions are indeed valid, *i.e.*, can a normal Michaelis–Menten type curve be drawn through the plateau areas within the limit of experimental error? The steps observed in Figure 2a and b appear to be unambiguous, but the first step observed in Figure 2c is much less pronounced. We have repeated the experiment presented in Figure 2c with each assay done in triplicate (approximately 120 points total). The error limits obtained in such a study did not invalidate the curve as presented.

The velocity–NAD^+ curves have been reproduced at least a dozen times with several different enzyme preparations over a period of 2 years. In order to avoid human bias in the results, at least four different individuals have reproduced the data without prior knowledge of the shape of the curve. Qualitatively similar results have been attained from yeast obtained from the Red Star Brand Co. in Milwaukee or from Detroit, and from yeast obtained from the Fleischman Co., locally. Results obtained with enzyme purified from Red Star Brand yeast by the rapid method of Stallcup *et al.* (1972) were essentially identical with those reported here. We are therefore confident that the results presented here are valid and are not negated by experimental error limits or human bias.

It should be pointed out that some loss of sensitivity has occurred in the mode of presentation of the data for publication. We have therefore included Hill plots of the data in order to emphasize the uniqueness of these results.

Any explanation of the kinetic results obtained must take into consideration the following observed properties of the enzyme. (1) The enzyme is believed to consist of four apparently identical subunits (Harris and Perham, 1965). (2) The enzyme binds, in theory, four molecules of NAD^+. The binding of NAD^+ to the enzyme isolated from Red Star Brand baker's yeast has been explained by a ligand induced sequential model (Koshland *et al.*, 1966) with subunit interactions which involve both positive and negative cooperative behavior (Cook and Koshland, 1970; Milne and Cook, 1974). The binding constants for NAD^+ therefore vary such that $K_1' < K_2' > K_3' > K_4'$ for the four subunits. (3) The enzyme binds 4 mol of cAMP/mol of enzyme with no apparent cooperativity observed. The binding of cAMP is inhibited by the presence of NAD^+ but the two ligands bind to different sites on the enzyme (Milne and Cook, 1974). (4) The binding of NAD^+ is also inhibited by the presence of cAMP, again noncompetitively. (5) No change in molecular weight of the enzyme occurs in the presence of NAD^+ and/or cAMP (Milne and Cook, 1974). (6) Biphasic binding of 5,5′-dithiobis(2-nitrobenzoate) and NAD^+ to the yeast enzyme has been observed, indicating at least two different conformational states of the enzyme or subunits (Chance and Park, 1967; Ellenrieder *et al.*, 1972). (7) Binding of 2 mol of sulfhydryl inhibitor to the yeast enzyme results in complete inactivation of enzyme activity, indicating this enzyme may exhibit half-site reactivity (Stallcup and Koshland, 1972, 1973a,b).

In the present results, in the absence of cAMP, the velocity–NAD^+ plots indicate a short intermediary plateau region at $5\text{–}10 \times 10^{-5}$ M NAD^+, which progressively disappears as G3P concentration is increased (Figure 1). Very importantly, however, the Hill plots of these data are biphasic at all concentrations of G3P, with a break occurring at approximately 40–50% saturation. This observation raises the possibility that the initial rate concentration plot up to the intermediary plateau region represents the activity of the first two subunits of the enzyme. This suggestion is supported by direct binding studies of NAD^+ which show similar Hill plots with a break at 50% saturation. The results presented in Figures 1 and 2a could therefore be explained by postulating that the binding of the first molecule of NAD^+ to the first subunit enhances the binding of the second molecule of NAD^+ to the second subunit, presumably through an induced conformational change. However, when the first two sites are near saturation, a more gross conformational change occurs, "opening up" the second pair of subunits which may bind NAD^+ and release product, *i.e.*, substrate activation. Conversely, the gross conformational change may make the third and fourth subunits less accessible to substrate, resulting in higher substrate concentrations to saturate the third and fourth sites, *i.e.*, negative cooperativity. Either of these proposals can explain the differential affinity of the enzyme for 5,5′-dithiobis(2-nitrobenzoate) and NAD^+. The observation that complete activity of the enzyme is destroyed when 2 mol of sulfhydryl are bound/mol of enzyme can be explained by assuming that the inhibitor binds irreversibly to the first two subunits, with the result that NAD^+ cannot open up the second two sites. In terms of negative cooperativity, the binding of two molecules of sulfhydryl inhibitor to the enzyme may cause a gross conformational change in the protein making the binding of substrate to the second two subunits so difficult that no activity is observed.

The results are consistent with the hypothesis that NAD^+ is the substrate responsible for the gross conformational changes observed. Glyceraldehyde 3-phosphate does not appear to cause any obvious conformational change influencing subunit interaction, since G3P appears to follow normal Michaelis–Menten kinetics (Figure 4). The presence of G3P must aid, in some way, the conformational changes produced by NAD^+, as indicated by the slow disappearance of the plateau region with increasing G3P concentration (Figure 1).

The presence of cAMP causes unusual kinetic effects in the velocity–NAD^+ curves (Figure 2) but has no effect other than inhibition on the velocity–G3P curves (Figure 4). Double reciprocal plots indicate that cAMP is a noncompetitive inhibitor of both NAD^+ and G3P, supporting the suggestion that cAMP binds to a different site on the enzyme than the catalytic site. Direct binding studies with cAMP (Milne and Cook, 1974) indicate that the cAMP binding sites are accessible on all four subunits in the absence of NAD^+, and no obvious subunit interaction is caused by cAMP binding. Importantly, however, the binding of cAMP does interfere with NAD^+ binding, presumably *via* a conformational change in the subunit. cAMP appears to inhibit the first 50% of the enzyme activity more dramatically than the second 50% at all concentrations of NAD^+ examined (Figure 5). This observation would support the general conclusion that two of the subunits of the enzyme behave differently, kinetically, than the other two.

Examining the data in Figure 2, one must assume that the

second pair of subunits, when available, are more active kinetically than the first two subunits. When enough cAMP is present to produce 25% inhibition, we would expect that on the average, approximately 1 mol of cAMP is bound/mol of enzyme. Since the presence of NAD^+ on the enzyme interferes with cAMP binding, it would seem logical that cAMP would prefer to bind to the fourth subunit which has been shown to bind NAD^+ poorly, if at all. The binding of cAMP would therefore result in poorer binding of NAD^+ to the fourth subunit. The results presented in Figure 2b are consistent with this hypothesis. A second intermediary plateau is observed presumably between the third and fourth binding sites, indicating that binding of NAD^+ to the fourth subunit has become even more difficult. When the cAMP concentration is increased to produce 50% inhibition, presumably 2 mol of cAMP, on the average, are bound to the enzyme. The presence of 2 mol of cAMP produces a third plateau region, presumably between the first and second binding sites for NAD^+ (Figure 2c). The velocity–NAD^+ curve therefore appears to be a simple titration curve of the four subunits. One would assume that this behavior is due to conformational changes in the subunits induced by cAMP binding with the result that NAD^+ binding is made more difficult, *i.e.*, increased negative cooperativity.

An obvious discrepancy between the binding results presented in the preceding paper (Milne and Cook, 1974) and the results presented in this paper are the n_H values obtained in the Hill plots of the data. One consistent feature of the NAD^+-binding and NAD^+–velocity curves is the step at 50% saturation, in the absence of cAMP. The binding curves exhibit slight positive cooperativity below 50% saturation (n_H = 1.45) and negative cooperativity above 50% saturation (n_H = 0.21). The NAD^+–velocity curves, however, exhibit little or no cooperativity below 50% saturation (n_H = 1.0–1.1) and positive cooperativity above 50% saturation (n_H = 2.2–2.5) (Figures 1b and 3a). In the presence of 0.15 mM cAMP, three n_H values were observed of 1.0, 2.3, and 3.7, respectively, *i.e.*, increasing positive cooperativity with increasing NAD^+ concentration (Figure 3b). If one extrapolates each step of the NAD^+–velocity curve back to zero concentration, it becomes obvious that a series of increasingly sigmoid lines would be obtained, with resultant n_H values greater than unity. The n_H values obtained are therefore misleading since the stepwise curves are typical of negative cooperative interactions. The purpose of including Hill plots in the present study has been to substantiate the presence of plateau regions, or steps, in the velocity–NAD^+ curves. The significance of the actual n_H values from the Hill plots remains to be determined.

This explanation appears to be consistent with the kinetic results and the binding results presented in the preceding paper. Of necessity, the explanation of these results has been teleological, since no precedence for such behavior has been observed previously. It is interesting to point out a study by Anderson and Weber (1965), who examined the binding of NADH to lactate dehydrogenase. A detailed fluorometric titration by these authors revealed similar complex behavior in the binding of NADH to beef muscle (M_4) lactate dehydrogenase, and were explained on the basis of relaxation effects.

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