Cosubstrate and Allosteric Modifier Activities of Structural Analogues of NAD and ADP for NAD-Specific Isocitrate Dehydrogenase from Bovine Heart†

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ABSTRACT: The specificity of bovine heart NAD-linked isocitrate dehydrogenase for the configurations of cosubstrate (NAD^+) and allosteric effector (ADP) was examined with 5 NAD^+ analogues modified in the adenosine portion and over 20 analogues of ADP altered in the purine ring, pentosyl group, and 5′-pyrophosphate group. NAD analogues in which the adenosine portion was replaced by inosine or 1,N^6-etheno-adenosine were inactive, but the formycin analogue had cosubstrate activity. Values of K_m for 2′-dNAD$^+$ and 3′-dNAD$^+$ were about five- to sevenfold larger than for NAD^+; V_{max} was about the same for 2′-dNAD$^+$ and NAD^+, and V_{max} was about one-fifth for 3′-dNAD$^+$ compared with NAD^+. The configuration or nature of substituents about carbons 2′ and 3′ of the ribosyl portion of ADP is not critical for allosteric activation since ADP analogues containing 2′-deoxy-D-ribose, 3′-deoxy-D-ribose, 2′,3′-dideoxy-D-ribose, 2′-*O*-methyl-D-ribose, and D-arabinose were about equally effective. α-ADP, where the glycosidic linkage is inverted, inhibits activation by ADP competitively. An unsubstituted 6-amino group and nitrogen 1 in ADP are essential for activity since N^6,N^6-dimethyl-aminopurine ribonucleoside 5′-pyrophosphate, 1-N^6-etheno-ADP, IDP, and 1-*N*-oxide-ADP were neither activators nor inhibitors. A hydrogen at carbon 2 of ADP is not essential for modifier activity since 2,6-diaminopurine nucleoside diphosphate was an activator; however, 2-hydroxy-6-aminopurine nucleoside diphosphate was inactive. Nitrogen 7 of ADP is not required for activity since the tubercidin analogue was as effective as ADP. The capability to bind to the allosteric site is retained when nitrogen 9 and carbon 8 of ADP are replaced by carbon and nitrogen, respectively, since the formycin analogue (FDP) was an inhibitor competitive with ADP. 8-Br-ADP was inactive. The secondary phosphates of the 5′-pyrophosphoryl group of ADP are required for activity since ADP-amide (P^1-(adenosine-5′)-P^2-aminopyrophosphate) was a positive modifier and 5′-adenylyl imidodiphosphate was an inhibitor competitive with ADP. Oxygen functions at the pyrophosphate group can be replaced by sulfur since ADP-α-S and ADP-β-S were equivalent to ADP as positive effectors. However, activity was lost when the β-phosphate group was replaced by sulfate (adenosine 5′-phosphosulfate) or when the bridge oxygen between the α- and β- or β- and γ-phosphates was replaced by a methylene group (α,β-methylene-ADP or β,γ-methylene-ATP).

NAD-specific isocitrate dehydrogenase (EC 1.1.1.41)

$$\text{D-isocitrate} + NAD^+ \rightarrow \alpha\text{-ketoglutarate} + CO_2 + NADH + H^+ \quad (1)$$

from animal tissues is an allosteric enzyme. The native enzyme from bovine heart of 320 000 daltons contains eight subunits (Giorgio et al., 1970), and recent evidence suggests that these eight subunits, though nearly identical in molecular weight for the enzymes from bovine and porcine heart, are composed of two (Ramachandran & Colman, 1978) or four (Rushbrook & Harvey, 1977, 1978) different polypeptide chains. Studies on the substrate specificity of the enzyme from bovine heart showed that *threo*-homoisocitrate and D-garcinia acid could serve as substrates for the enzyme in place of D-isocitrate (Plaut et al., 1975) and that 3-acetylpyridine-AD, 3-pyridine aldehyde-AD, or thionicotinamide-AD was utilized as a cosubstrate for the bovine heart dehydrogenase; however, replacement of the adenine portion of NAD^+ by hypoxanthine led to loss of activity (Chen & Plaut, 1963).

ADP is a positive modifier for the enzyme from a number of mammalian tissues (for review, see Plaut, 1970). ADP lowers the K_m of the substrate magnesium isocitrate, but does not change V_{max} (Plaut et al., 1974). In attempts to define structural specificity, ADP could not be replaced by AMP, IDP, GDP, UDP, or CDP; however, 2′-dADP was a positive modifier (Chen & Plaut, 1963; Plaut & Aogaichi, 1968).

In the present studies with homogeneous NAD-specific isocitrate dehydrogenase from bovine heart, analogues of NAD^+ altered in the adenosine portion and ADP analogues modified in the purine ring, the ribosyl group and the 5′-pyrophosphate group were examined to describe more completely the cosubstrate and effector sites of the enzyme.

Experimental Procedures

Chemicals. ADP, 2′-dADP,[1] 5′-ADP, and NAD^+ were purchased from Sigma. ε-ADP, ε-CDP, 5′-*ara*-ADP, 2-*O*-methyl-ADP, AP-CH_2-P, APP-CH_2-P, dd-ATP, NID^+, and

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[1] Abbreviations used: NID^+, 2′-dNAD$^+$, 3′-dNAD$^+$, ε-NAD^+, and NFD^+, analogues in which the adenosine group of NAD^+ has been replaced by inosine, 2′-deoxyadenosine, 3′-deoxyadenosine, 1,N^6-ethenoadenosine, and formycin, respectively; 2′-dADP, 2′-deoxy-ADP; 3′-dADP, 3′-deoxy-ADP; dd-ADP, 2′,3′-dideoxy-ADP; ε-ADP, 1,N^6-etheno-ADP; ε-CDP, 1,N^4-etheno-CDP; FDP, 5′-formycin diphosphate; TuDP, 5′-tubercidin diphosphate; 5′-*ara*-ADP, 5′-adenine-9-β-D-arabinosyl diphosphate; α-ADP, 5′-adenine-9-α-D-ribosyl diphosphate; 2-NH_2-ADP, 2,6-diaminopurine ribonucleoside 5-diphosphate; 2-OH-ADP, 2-hydroxy-6-aminopurine ribonucleoside 5′-diphosphate; N^6-dimethyl-ADP, N^6,N^6-dimethylaminopurine ribonucleoside 5′-diphosphate; ADP-amide (or 5′-APP-NH_2), P^1-(adenosine-5′)-P^2-aminopyrophosphate; 2′-*O*-methyl-ADP, 2′-*O*-methyladenosine 5′-diphosphate; 1-*N*-oxide-ADP, adenosine 1-oxide 5′-diphosphate; 8-Br-ADP, 8-bromoadenosine 5′-diphosphate; ADP-α-S, adenosine 5′-(O^1-thiodiphosphate); ADP-β-S, adenosine 5′-(O^2-thiodiphosphate); 5′-APP-NH-P, 5′-adenylyl imidodiphosphate; 5′-APS, adenosine 5′-phosphosulfate; AP-CH_2-P, α,β-methylene-ADP; APP-CH_2-P, β,γ-methylene-ATP.

ϵ-NAD^+ were from P-L Biochemicals. ADP-β-S, adenosine 5′-phosphorothioate, and 5′-APP-NH-P were from Boehringer Mannheim. 2,6-Diaminopurine nucleoside, adenine 9-β-D-arabinoside, and N^6,N^6-dimethylaminoadenosine were from Vega-Fox. Adenine 9-α-D-ribofuranoside was from Calbiochem. Formycin was from Meija Keika Kaisha, Ltd., and tubercidin from Upjohn Laboratories. Samples of ADP-amide and 2-OH-ADP were gifts from Boehringer Mannheim and Dr. O. Bârzu, respectively. Dr. D. Dennis kindly provided a sample of ribose 5-pyrophosphate. DL-*threo*-Isocitrate lactone from Sigma was recrystallized from ethyl butyrate and hydrolyzed with alkali to isocitrate before use.

Synthesis of Analogues of NAD^+ and ADP. 2′-$dNAD^+$, 3′-$dNAD^+$, and NFD^+ were prepared as described previously (Suhadolnik et al., 1977). 8-Br-ADP formed by bromination of ADP by the procedure of Mumeyama et al. (1971) for bromination of adenosine was purified by precipitation as the barium salt followed by chromatography on a Dowex AG 1-X8 (200–400 mesh) formate column. 1-*N*-Oxide-ADP was prepared by oxidation of ADP with monopermaleic acid and purified as described by Mantsch et al. (1975). 2-OH-ADP was prepared by irradiation of thin layers of solutions of 1-*N*-oxide-ADP with a mercury arc lamp and purified on a column of Dowex AG 1-X8 (200–400 mesh) carbonate with a linear gradient (0.01–1 M) of ammonium bicarbonate (Mantsch et al., 1975). The spectral, chromatographic, and enzymatic properties of 2-OH-ADP prepared in this laboratory agreed with those of a sample generously furnished by Dr. O. Bârzu. ADP-α-S was synthesized from adenosine 5′-phosphorothioate as described by Eckstein & Goody (1976). ADP-amide was prepared by enzymatic hydrolysis of 5′-APP-NH-P with *E. coli* alkaline phosphatase (Yount et al., 1971b) followed by purification on a column of Dowex AG 1-X8 (200–400 mesh) carbonate with a linear gradient (0.01–1 M) of ammonium bicarbonate. The spectral and chromatographic properties of ADP-amide prepared in this laboratory agreed with a sample kindly provided by Boehringer Mannheim. 2′,3′-Dideoxyadenosine 5′-triphosphate (dd-ATP) was converted to the diphosphate (dd-ADP) with glucose and yeast hexokinase, and dd-ADP was purified by column chromatography.

FDP, TuDP, 5′-*ara*-ADP, N^6-dimethyl-ADP, 3′-dADP, α-ADP, and 2-NH_2-ADP were prepared by chemical synthesis from each of the appropriate nucleosides. The initial phosphorylation of nucleoside (0.8–1.6 mmol) with phosphorus oxychloride in triethyl phosphate to the corresponding 5′-nucleoside monophosphate was done by a modification of the method of Sowa & Ouchi (1975). The 5′-nucleoside monophosphates were separated from unreacted nucleosides on a 1.7 × 12 cm column of XAD-4 by elution with water (Uematsu & Suhadolnik, 1976) followed by chromatography on a 1.6 × 31 cm column of Dowex AG 1-X8 (200–400 mesh) formate. After an initial wash with water, the 5′-nucleoside monophosphates were brought off the column with 0.75 M formic acid. The purity of 5′-nucleoside monophosphates was examined by thin-layer chromatography and the location of the phosphate at the 5′ position was determined by hydrolysis with *Crotalus adamanteus* 5′-nucleotidase. The 5′-nucleoside diphosphates were prepared from the 5′-nucleoside monophosphates by the method of Moffatt & Khorana (1961) and purified by chromatography on a 1 × 15 cm column of Dowex AG 2-X10 (200–400 mesh) chloride with a linear gradient (0–0.1 M, 1200 mL) of lithium chloride in 0.003 M HCl. The column effluents were lyophilized and the lithium salts of the nucleoside diphosphates were recovered by precipitation with acetone from methanol solution. The purity of the products was checked by analyses for total and acid-labile phosphorus, spectra, and thin-layer chromatography.

Thin-Layer Chromatography. A number of systems of thin-layer chromatography were used to examine the purity of nucleosides and nucleoside mono- and diphosphates. On Eastman or Avicel cellulose thin-layer supports, the solvent systems were: (I) 1-propanol:concentrated NH_4OH:H_2O (6:3:1); (II) isobutyric acid:1 M NH_4OH:0.1 M Na_4EDTA (250:150:4); (III) 0.5 M KH_2PO_4; (IV) 2-propanol:concentrated NH_4OH:H_2O (7:1:2). On PEI-cellulose support (Polygram Cel-300) (Macherey and Nagel Co.), the solvent systems were: (V) 0.5 M KH_2PO_4; (VI) 1 M LiCl.

Enzyme Assays and Calculations. NAD-specific isocitrate dehydrogenase was purified to homogeneity and stored as described previously (Giorgio et al., 1970).

Initial velocities were determined by following the reduction of NAD^+ and its analogues at 340 nm in 1-cm lightpath silica cells in a Gilford Model 240 spectrophotometer set at 0.02 Å for full recorder expansion. The incubations were performed at 25 °C.

For determination of the kinetic constants of NAD^+ and its analogues, the composition of incubation media was as follows: 5.3 mM DL-isocitrate, 1.3 mM $MnSO_4$, 0.67 mM ADP, 167 mM Na–Hepes at pH 7.4 and varying quantities of NAD^+ analogues. Under these conditions, the calculated concentration of manganous DL-isocitrate was 1.06 mM (Grzybowski et al., 1970).

For determination of the kinetic constants of ADP and its analogues, the basic composition of incubation media was as follows: 24.3 mM DL-isocitrate, 0.27 mM $MgSO_4$, 0.50 mM NAD^+ and 167 mM Na-Hepes at pH 7.4. Under these conditions the calculated concentrations of magnesium DL-isocitrate and free Mg^{2+} were 0.25 mM and 0.02 mM, respectively (Grzybowski et al., 1970; Plaut et al., 1974). Varying quantities of ADP or its analogues were added to the basal medium with appropriate additional quantities of magnesium to compensate for chelation of added nucleotides. It was assumed for the latter that the ADP analogues have the same stability constant as ADP with magnesium.

The nature of initial velocity curves was determined by graphical analysis and then analyzed by fitting to the appropriate computer programs developed by Cleland (1963). Data which give linear double-reciprocal plots of initial velocity against NAD^+ and NAD^+ analogue concentration were fitted to eq 2.

$$v = VA/(K + A) \tag{2}$$

Plots which were rectangular hyperbolas, but did not pass through the origin in plots of v against ADP and ADP analogues, were fitted to eq 3

$$v = v_0\left[\frac{1 + \frac{\text{ADP}}{K_N}}{1 + \frac{\text{ADP}}{K_D}}\right] \tag{3}$$

where v_0 is the initial velocity of the reaction in the absence of allosteric effector. The maximal velocity will be given by setting ADP or ADP analogue $\rightarrow \infty$, in which case

$$V_{max} = v_0\left[\frac{K_D}{K_N}\right] \tag{4}$$

The K_m for ADP or ADP analogues is given by the relationship $K_m = K_D$.

Table I: Effect on Activity of NAD-Isocitrate Dehydrogenase of Modification of Adenosine Portion of the Effector ADP and Cosubstrate NAD^+

	ADP analogue[a]		NAD analogue[b]	
substituent	rel K_m ratio[i]	velocity enhancement ratio[j]	rel K_m ratio[k]	rel velocity ratio[l]
adenosine	1.0[c]	4.9[c]	1.0[c]	1.0
inosine		1.0[d]	∞	0.00[e]
$1,N^6$-*etheno*-adenosine		1.0	∞	0.00[f]
formycin		inhibitor	2.9;[b] 3.8[g]	0.35;[b] 0.27[g]
2′-deoxyadenosine	1.0	4.0	8.4;[b] 6.6[h]	1.2;[b] 1.2[h]
3′-deoxyadenosine	1.7	5.6	4.7[h]	0.19[h]

[a] Reaction at pH 7.4; concentrations of magnesium DL-isocitrate and free Mg^{2+} are 0.25 and 0.02 mM, respectively. [b] Reaction at pH 7.2; 5.3 mM DL-isocitrate, 1.3 mM $MnSO_4$, and 0.67 mM ADP. [c] K_m of NAD^+ was 0.07 ± 0.02 mM in the absence and presence of ADP. The values of K_m(app) for ADP ranged from 0.05 to 0.5 mM and the velocity enhancement caused by ADP varied from 2.7 to 5.8 (see the text). [d] At 0.2 mM nucleotide. [e] No activity when tested between 0.5 and 6.0 mM NID^+ with 2.5 mM magnesium DL-isocitrate, 0.1 mM free Mg^{2+}, and 0.12 mM ADP at pH 7.4. Also, there was no inhibition by 6.0 mM NID^+ under these conditions of reduction rate of NAD^+ (0.20 mM). [f] No activity when tested with up to 0.64 mM ϵ-NAD^+. [g] The incubation conditions were the same as under *b*, except that ADP was absent. [h] The reaction mixture contained 2.5 mM magnesium DL-isocitrate, 0.1 mM free Mg^{2+}, and 0.12 mM ADP at pH 7.4. [i] Ratio of K_m^{analogue}(app)/K_m^{ADP}(app). [j] Ratio of $V_{+\text{nucleotide}}/V_{-\text{nucleotide}}$. [k] Ratio of $K_m^{\text{analogue}}/K_m^{NAD^+}$. [l] $V_m^{\text{analogue}}/V_m^{NAD^+}$.

When inhibition was indicated by the results, initial velocity experiments with substrate varied at a series of fixed inhibitor concentrations were fitted to eq 5–7 describing competitive, uncompetitive, or noncompetitive inhibition, respectively.

$$v = \frac{VA}{K(1 + I/K_{is}) + A} \quad (5)$$

$$v = \frac{VA}{K + (1 + I/K_{ii})A} \quad (6)$$

$$v = \frac{VA}{K(1 + I/K_{is}) + (1 + I/K_{ii})A} \quad (7)$$

In cases where the double-reciprocal plots of such data intersected near the ordinate, the data were fitted to both eq 5 and 7 to distinguish competitive from noncompetitive inhibition.

Results

Different assay conditions were selected to compare the effectiveness of analogues of NAD^+ and of ADP as cosubstrates and allosteric modifiers, respectively. With NAD^+ analogues, the reaction mixtures contained 0.67 mM ADP and 1.06 mM manganous DL-isocitrate or 2.5 mM magnesium DL-isocitrate, conditions which are nearly saturating for substrate (Fan et al., 1975). Tests for modulation of enzyme activity by ADP analogues were done with a constant nonsaturating concentration of magnesium isocitrate (0.25 mM) and nearly saturating NAD^+ (0.5 mM) (cf. Table I where K_m of NAD^+ is 0.07 mM). With changing ADP analogue concentration, the total magnesium concentration was adjusted to maintain constant free Mg^{2+} (0.02 mM) by using the calculations and stability constants reported previously (Plaut et al., 1974). This minimized complications due to inhibition by free Mg^{2+} and changes in the ratios of chelated and free forms of the nucleotides. Free ADP has been shown to be the activator of the enzyme from heart (Plaut et al., 1974). Under these incubation conditions, the values of K_m for NAD^+ analogues should approach those of limiting K_m, whereas the constants for enhancement of activity by nucleoside diphosphates are K_m(app) and are valid only for comparing the relative allosteric effects of these nucleotides. The same applies to values of K_{is} for compounds which are inhibitors competitive with ADP.

Analogues of NAD^+ and ADP. The effect of the same modification in the adenosine portion both of ADP and NAD^+ is shown in Table I. For comparison of K_m of each appropriate analogue with NAD^+ and ADP, respectively, the kinetic constants are expressed as the ratios, i.e., relative K_m. Velocity enhancement in the case of ADP analogues refers to the ratio of the calculated velocity when the nucleotide concentration is infinite (v) compared with the rate in the absence of nucleotide (v_0). With NAD^+ analogues, the term relative velocity indicates the ratio of the maximal velocity with analogue compared with that with NAD^+. The ranges of kinetic constants reported represent the means of at least two experiments; in each trial, the appropriate analogue was accompanied by a parallel experiment with NAD^+ or ADP.

Replacement of the 6-amino group of the adenine moiety by a hydroxyl group as in inosine derivatives or of covalently binding the 6-amino and 1-nitrogen groups as in $1,N^6$-ethenoadenosine derivatives (Secrist et al., 1972a,b; Kotchetkov et al., 1971) led to loss of regulator and cosubstrate activity of ADP and NAD analogues (Table I), suggesting that the free 6-amino group is required for activity at either site. With formycin analogues, where the 6-amino group is retained but the imidazole portion of adenosine is replaced by a pyrazole ring, the formycin NAD^+ analogue had cosubstrate activity with values of K_m three- to fourfold higher and V_{max} about one-third of that of the corresponding kinetic constants for NAD^+. However, formycin diphosphate was an inhibitor rather than a positive modulator of the enzyme. The results with the formycin analogues (Table I) extend earlier kinetic observations that ADP does not bind to the cosubstrate binding site as indicated by a lack of competitive reversal by ADP of NADH inhibition (Plaut & Aogaichi, 1968) and suggests that the adenine binding sites for modifier and cosubstrate differ in certain details.

Substitution by hydrogen of the 2′-hydroxyl or 3′-hydroxyl group in the ribosyl group of the adenosine moiety resulted in analogues with modulator activity quite similar to that of ADP, as judged by K_m(app) and enhancement of reaction velocity. However, the 2′-deoxy- and 3′-deoxyadenosine analogues of NAD^+ have five to seven times higher values of K_m than NAD^+. The maximal velocities for 2′-dNAD^+ and NAD^+ were about the same, while the relative velocity of 3′-dNAD^+ was about one-fifth of that of NAD^+ (Table I).

The large K_m of the deoxy-NAD^+ preparations is not due to contamination by NAD^+, judged by the chromatographic separation steps used in their preparation and analysis of the final products on several systems of TLC. Furthermore, 2′-dATP and 3′-dATP, used for the synthesis of the corresponding 2′-dNAD^+ and 3′-dNAD^+, were treated with periodate which would have cleaved the ribosyl moiety of contaminating ATP before subsequent further purification by chromatography.

Modulator Activity of Analogues of ADP. Since there tend to be significant variations in allosteric activation with different enzyme preparations and with enzymes stored for varying periods of time, each experiment with a particular analogue (Tables II–IV and Figures 1–4 and 6) was accompanied by a control experiment with ADP. The constants for ADP

Table II: Effector Activity of Analogues of ADP Modified in the Ribosyl Group[a]

nucleotide	concn range (mM)	enzyme no.	K_m(app) of nucleotide (mM)	velocity enhancement[b] ratio
2′-deoxy-ADP	0.05–1.0	1	0.271 ± 0.034	4.0
ADP	0.05–1.0	1	0.273 ± 0.038	5.3
3′-deoxy-ADP	0.05–0.25	1	0.515 ± 0.035	5.2
ADP	0.10–0.50	1	0.186 ± 0.018	3.5
D-*ara*-ADP	0.19–0.96	1	0.224 ± 0.035	4.4
ADP	0.15–1.9	1	0.177 ± 0.015	4.6
2′-*O*-methyl-ADP	0.18–0.88	2	0.428 ± 0.133	5.5
ADP	0.20–1.00	2	0.544 ± 0.078	5.8
2′,3′-dideoxy-ADP	0.039–0.15	3	0.051 ± 0.011	2.9
ADP	0.040–0.16	3	0.067 ± 0.007	2.7
α-ADP vs. ADP	0.57–1.15	1	0.792 ± 0.098[c]	inhibitor
ADP	0.16–0.48	1	0.173 ± 0.025	3.5

[a] Assayed at pH 7.4 with 0.25 mM magnesium DL-isocitrate, 0.02 mM free Mg^{2+}, and 0.5 mM NAD^+. [b] Ratio of $[(v - v_0)_{max} + v_0]/v_0$. v and v_0 are velocities in the presence and absence of nucleotides, respectively. [c] K_i value.

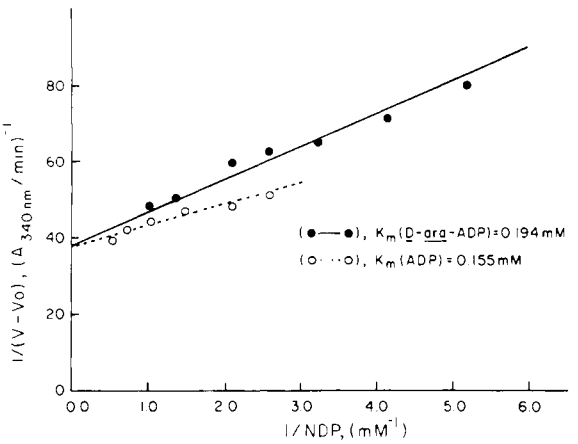

FIGURE 1: Comparison of the allosteric activities of D-*ara*-ADP and ADP. The conditions of incubation and the synthesis of D-*ara*-ADP are described under Experimental Procedures.

obtained in the same experiment where the ADP analogue was tested are shown in all cases where such a comparison becomes significant. While the kinetic constants varied appreciably between experiments, the ratios of allosteric effects of active nucleoside diphosphate analogues compared with ADP were relatively constant.

Variations in the Ribosyl Group of ADP. The effective modulation of enzyme activity by 2′-dADP and 3′-dADP (Tables I and II) encouraged us to examine the effect of alteration of the ribosyl group in more detail. In three experiments with 3′-dADP, K_m(app) ranged from 0.17 to 0.58 mM with a mean of 0.42 mM; the mean values of relative K_m(app) and velocity enhancement were 1.7 and 5.6, respectively.

The results with other analogues are shown in Table II and indicate that there is considerable latitude in modification of the ribosyl group of ADP on effector activity. Thus, when the 2′-hydroxyl group was inverted as in D-arabinosyl-ADP (D-*ara*-ADP) or when the hydrogen of the 2′-hydroxyl group was replaced by a methyl group as in 2′-*O*-methyl-ADP, the allosteric activation was almost as effective as with ADP (Table II). The experiments with D-*ara*-ADP shown in Table II and Figure 1 were done with preparations synthesized in this laboratory and purchased from a commercial supplier, respectively. In four experiments with D-*ara*-ADP, K_m(app) ranged from 0.19 to 0.57 mM with a mean of 0.31 mM; the mean values of relative K_m(app) and velocity enhancement were 1.1 and 4.3, respectively. The averages of two experiments with 2′-*O*-methyl-ADP presented in Table II show values of relative K_m(app) and velocity enhancement of 0.79 and 5.5, respectively. Activation was essentially the same as with ADP when both hydroxyls at carbons 2′ and 3′ of the pentosyl group of ADP were replaced by hydrogen. With 2′,3′-dideoxy-ADP, the values of relative K_m(app) and velocity enhancement were 0.76 and 2.9, respectively (Table II).

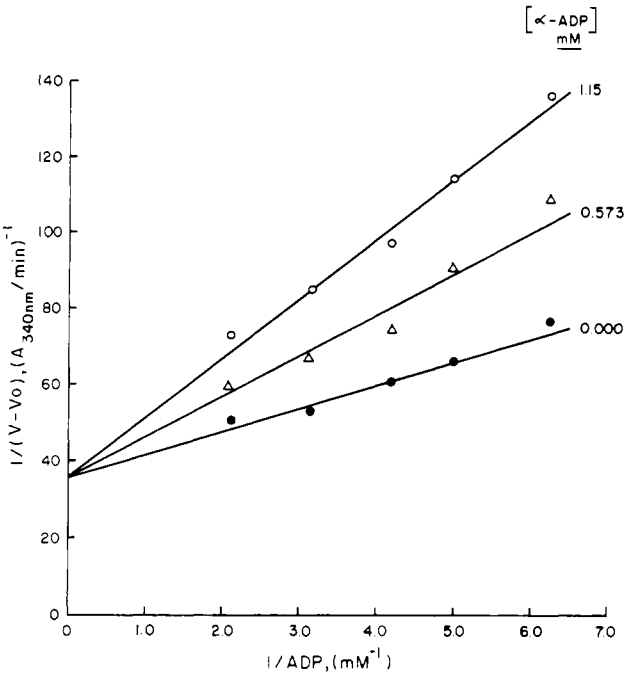

FIGURE 2: Inhibition by α-ADP of positive modulation by ADP (β-ADP). The concentration of ADP was varied between 0.160 and 0.480 mM at fixed concentrations of 0.000, 0.573, and 1.145 mM α-ADP.

Inversion at the anomeric carbon of the ribosyl group of ADP resulted in loss of positive modifier activity; nevertheless, α-ADP appears to be bound at the allosteric site since it is an inhibitor competitive with ADP (Table II and Figure 2). In three experiments, with fixed levels of α-ADP ranging from 0.34 to 1.15 mM and ADP (i.e., β-ADP) varied from 0.056 to 0.48 mM, the ratio of K_{is} for α-ADP to K_m(app) for ADP ranged from 3.9 to 4.8. Analysis of the data obtained in these experiments shows a better fit to competitive (eq 5) than to noncompetitive (eq 7) inhibition. α-ADP does not bind at the cosubstrate site since no inhibition was obtained with 0.34–1.13 mM α-ADP when magnesium DL-isocitrate was nearly sat-

Table III: Effector Activity of Analogues of ADP Modified in the Purine Group[a]

nucleotide	concn range (mM)	K_m(app) of nucleotide (mM)	velocity enhancement[b] ratio
2-NH_2-ADP	0.21–0.61	0.307 ± 0.044	4.2[c]
ADP	0.12–0.67	0.198 ± 0.018	5.4
2-NH_2-ADP	0.15–0.51	0.255 ± 0.033	3.4[d]
ADP	0.15–0.67	0.129 ± 0.029	3.3
TuDP	0.04–0.16	0.067 ± 0.005	3.1
ADP	0.04–0.16	0.061 ± 0.012	2.8
FDP vs. ADP	0.087–0.17	0.242 ± 0.039[g]	inhibitor
ADP	0.16–0.48	0.197 ± 0.028	4.7
2-OH-ADP	0.41–1.0		0.96[e]
2-OH-ADP + 0.16 mM ADP	0.66–2.64		0.94[f]
1-*N*-oxide-ADP	0.23–1.16		1.0[e]
1-*N*-oxide-ADP + 0.16 mM ADP	0.23		0.99[f]
ϵ-ADP	0.23–0.57		1.0[e]
ϵ-ADP + 0.04 mM ADP	0.23–0.57		0.94[f]
N^6-Me-ADP + 0.12 mM ADP	1.9–9.5		0.5[f]
ϵ-CDP	0.2–0.4		0.94[e]
8-Br-ADP	0.2		1.0[e]
ribose-5-PP	0.14–0.56		0.87[e]

[a] Assayed at pH 7.4 with 0.25 mM magnesium DL-isocitrate, 0.02 mM free Mg^{2+}, and 0.5 mM NAD^+. [b] Ratio of $[(v - v_0)_{max} + v_0]/v_0$. v and v_0 are velocities in the presence and absence of nucleotides, respectively. [c] 2-NH_2-ADP prepared in this laboratory. [d] 2-NH_2-ADP from P-L Biochemicals. [e] Ratio of velocity in the presence of analogue compared with velocity in the absence of analogue. The value given is for the largest concentration of analogue. [f] As in footnote *e*, except that ADP was present, in the absence and presence of analogue. In absence of analogue, the ratios of velocity in the presence of ADP (v) compared with the velocity in the absence of ADP (v_0) were v/v_0 = 1.5–1.8 when ADP varied from 0.12 to 0.16 mM. In the experiment where the effect of ϵ-ADP was tested, v/v_0 was 1.6 with 0.04 mM ADP. [g] K_i value.

urating (2.5 mM) and NAD^+ was varied from 0.13 to 0.25 mM (results not shown).

Substitution of the Purine Group of ADP. It was observed in earlier studies (Chen & Plaut, 1963) that IDP, GDP, CDP, and UDP did not replace ADP as an allosteric effector of NAD-specific isocitrate dehydrogenase, suggesting that a nucleoside diphosphate with a free 6-amino substituent at the purine base is required for activity. This seems consistent with the present observation (Table III) that the etheno derivatives ϵ-ADP and ϵ-CDP, which have been reported to replace adenosine nucleotides in a number of phosphotransferase reactions (Secrist et al., 1972a,b), are neither activators nor inhibitors of the enzyme. N^6-Dimethyl-ADP (N^6,N^6-dimethylaminopurine nucleoside diphosphate) was not an activator of the enzyme. Under the conditions of subsaturating substrate concentrations used in the experiment shown in Table III (0.25 mM magnesium DL-isocitrate), N^6-dimethyl-ADP was a weak inhibitor (K_i = 5.5–6.5 mM) when ADP (0.12 mM) was present but not when magnesium isocitrate was nearly saturating (2.5 mM). Ribose 5-pyrophosphate (Horecker et al., 1957) which does not contain a base at the anomeric carbon is not an activator, but is an inhibitor at high concentration—probably due to chelation of magnesium.

However, substituents other than a free 6-amino group at the purine moiety of a nucleoside diphosphate also affect

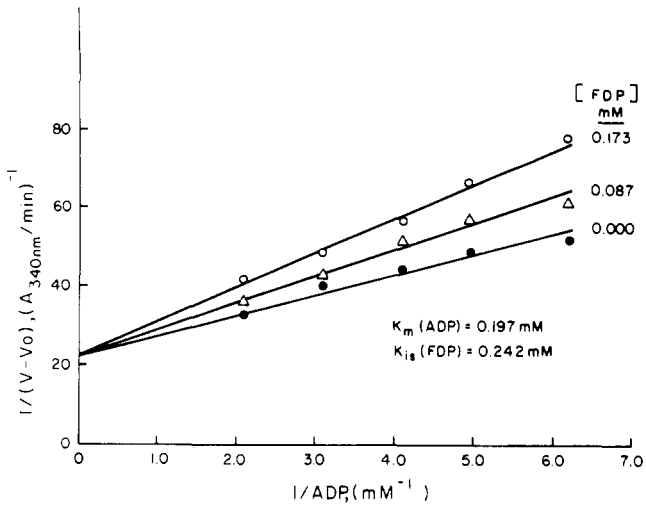

FIGURE 3: Inhibition by formycin diphosphate (FDP) of positive modulation by ADP. The concentration of ADP was varied between 0.17 and 0.48 mM at fixed concentrations of 0.00, 0.087, and 0.173 mM FDP. The conditions of incubation and calculations are described under Experimental Procedures.

modifier activity for NAD-isocitrate dehydrogenase. Thus, 8-bromo-ADP, 1-*N*-oxide-ADP, and 2-OH-ADP (2-hydroxy-6-aminopurine nucleoside diphosphate) were not positive effectors (Table III). 1-*N*-Oxide-ADP has been reported by a number of investigators to replace ADP in certain phosphotransferase reactions (Cramer et al., 1963; Mantsch et al., 1975); however, it was inactive as a modifier for bovine liver glutamate dehydrogenase (Mantsch et al., 1975) and it was neither an activator nor inhibitor for isocitrate dehydrogenase (Table III). On the other hand, 2-OH-ADP (and 2-OH-ATP) has been reported by Mantsch et al. (1975) to be a potent inhibitor of glutamate dehydrogenase. The 2-hydroxy-6-aminopurine nucleotides are structural analogues of both guanosine and adenosine nucleotides and it remains to be established which of the interactions of the natural nucleotides with glutamate dehydrogenase (Goldin & Frieden, 1971) is affected by the analogue(s). 2-OH-ADP was not a positive effector for isocitrate dehydrogenase. The small inhibition observed with this compound at higher concentrations (Table III) appears not to be due to binding at the allosteric site of the enzyme since it could not be reversed by ADP.

Although 2-OH-ADP was inactive, substitution at carbon 2 of ADP per se does not obliterate activity, since 2-NH_2-ADP (2,6-diaminopurine nucleoside diphosphate) was an allosteric modulator. In the experiments shown in Table III, the mean values of relative K_m(app) and velocity enhancement were 1.8 and 3.8, respectively.

Tubercidin diphosphate (TuDP) and formycin diphosphate (FDP), in which the imidazole portion of adenine of ADP is replaced by a pyrrole and pyrazole ring, respectively, bind at the allosteric site of NAD-isocitrate dehydrogenase. TuDP was an allosteric effector about equivalent to ADP in activity (Table III) and the C-glycoside FDP was an inhibitor competitive with ADP (Figure 3). The bindings of FDP and ADP to the enzyme occur with about equal affinities with a ratio of K_{is} for FDP to K_m(app) for ADP of 1.2 (Table III).

Modification of the 5'-Pyrophosphate Group of ADP. Earlier studies with NAD-isocitrate dehydrogenase from bovine heart (Chen & Plaut, 1963) had shown that AMP (or a combination of AMP and inorganic phosphate[2]) did not

[2] T. Aogaichi and G. W. E. Plaut, unpublished observation.

Table IV: Effector Activity of Analogues of ADP Modified in the 5′-Pyrophosphoryl Group[a]

nucleotide	concn range (mM)	K_m(app) of nucleotide (mM)	velocity enhancement[b] ratio
ADP-amide	0.12–1.0	0.460 ± 0.045	3.1[c]
ADP	0.12–0.48	0.129 ± 0.016	3.7
ADP-α-S	0.14–0.57	0.128 ± 0.030	4.7
ADP	0.12–0.50	0.191 ± 0.034	4.6
ADP-β-S	0.12–0.87	0.116 ± 0.013	3.1
ADP	0.16–0.96	0.083 ± 0.008	3.2
5′-APS	0.2–1.0		1.0[d]
5′-APP-NH-P vs. ADP	0.15–0.38	0.118 ± 0.030[f]	inhibitor
ADP	0.24–0.12	0.189 ± 0.038	
AP-CH_2-P	0.7		0.83[d]
AP-CH_2-P + 0.7 mM ADP	0.7		1.1[e]
APP-CH_2-P	0.7		0.96[d]
APP-CH_2-P + 0.7 mM ADP	0.7		1.1[e]

[a] Assayed at pH 7.4 with 0.25 mM magnesium DL-isocitrate, 0.02 mM free Mg^{2+}, and 0.5 mM NAD^+. [b] Ratio of $[(v - v_0)_{max} + v_0]/v_0$. v and v_0 are velocities in the presence and absence of nucleotides, respectively. [c] ADP-amide was prepared as described under Experimental Procedures. [d] See footnote *e* of Table III. [e] See footnote *f* of Table III. [f] K_i value.

affect the activity of the enzyme; ATP and ADPR were inhibitors, but the inhibition was competitive with NAD^+ and ATP displaced bound NADH from the enzyme (Harvey et al., 1972).

A number of analogues in which an oxygen function at the pyrophosphoryl group of ADP was replaced by sulfur or an amide group affected the activity of the enzyme (Table IV). Murray & Atkinson (1968) had reported that adenosine 5′-phosphorothioate was as effective as AMP for the activation of yeast NAD-isocitrate dehydrogenase. We now find that ADP-α-S and ADP-β-S have almost the same modulator activity as ADP for bovine heart NAD-isocitrate dehydrogenase as judged by the kinetic constants (Table IV). ADP-α-S synthesized by the method of Eckstein & Goody (1976) contains two diastereoisomers which exhibit different activities with certain phosphotransferases. The unresolved substance was used in the present experiments and it is not known whether the isomers of ADP-α-S differ in allosteric activation of NAD-isocitrate dehydrogenase.

ADP-amide in which one OH at the β-phosphate of ADP is replaced by NH_2 was an allosteric effector. The value of relative K_m was 3.6 and the velocity enhancement was only slightly lower than that for ADP (Table IV). 5′-Adenylyl imidodiphosphate (5′-APP-NH-P) in which the β-phosphate of ADP is substituted with an imidophosphate group was an inhibitor competitive with ADP. In the experiments shown in Table IV and Figure 4A, the mean values for K_{is} of 5′-APP-NH-P and K_m(app) for ADP were 0.11 mM and 0.15 mM, respectively. Experiments by Yount et al. (1971a) indicated that 5′-APP-NH-P is a potent inhibitor competitive with ATP for meromyosin ATPase. Since inhibition by ATP of NAD-isocitrate dehydrogenase is competitive with NAD^+ (Chen & Plaut, 1963; Plaut & Aogaichi, 1968), experiments were done in the absence and presence of 5′-APP-NH-P with incubation mixtures with varied NAD^+ but no ADP. In the experiment shown in Figure 4B, with a nearly saturating concentration of magnesium isocitrate (2.5 mM), there was almost no inhibition by 5′-APP-NH-P at any level of NAD^+.

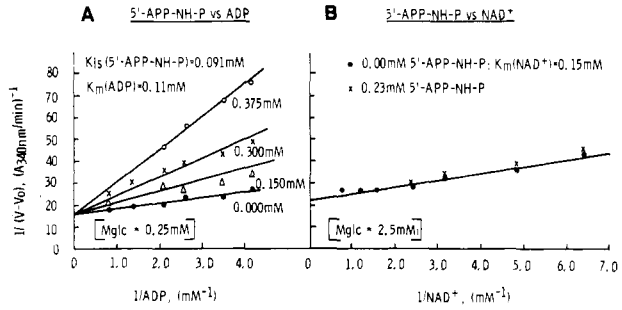

FIGURE 4: Inhibition by 5′-adenylyl imidodiphosphate (5′-APP-NH-P). (A) The concentration of ADP was varied between 0.24 and 1.21 mM at fixed concentrations of 0.00, 0.150, 0.300, and 0.375 mM 5′-APP-NH-P. The concentration of magnesium DL-isocitrate (MgIc) was 0.25 mM. (B) The concentration of NAD^+ was varied from 0.16 to 1.27 mM in the absence and presence of 5′-APP-NH-P (0.23 mM). The concentration of magnesium DL-isocitrate was 2.5 mM and ADP was absent.

This level of 5′-APP-NH-P (0.23 mM) was inhibitory at a nonsaturating concentration of magnesium isocitrate (0.25 mM) and with nearly saturating NAD^+ (0.5 mM); this is particularly apparent at low concentrations of ADP (e.g., 0.25 mM in Figure 4A). The results in Figure 4 indicate that 5′-APP-NH-P competes with ADP at the regulatory site and not with NAD^+ at the cosubstrate site; furthermore, the effect of magnesium isocitrate concentration on extent of inhibition by 5′-APP-NH-P in absence of ADP suggests a kinetic competitive relationship between substrate and the inhibitory ADP analogue. The latter would be expected since it was shown previously that the apparent K_m values of magnesium isocitrate declined with increasing ADP concentration and K_m(app) for ADP was dependent on substrate concentration (Plaut et al., 1974). Reversal of 5′-APP-NH-P inhibition by magnesium isocitrate was observed in experiments (not shown) where NAD^+ was constant (0.5 mM) and substrate was varied at various fixed levels of 5′-APP-NH-P. Values of K_{is} for 5′-APP-NH-P were between 0.2 mM and 0.4 mM; however, the data were not precise enough to prove that the inhibition was truly competitive. The high stability constant of magnesium 5′-APP-NH-P (38.2 mM^{-1}) (Yount et al., 1971b) compared with that for magnesium isocitrate (0.521 mM^{-1}) poses the technical problem of maintaining a constant ratio of the chelated and free forms of 5′-APP-NH-P, while levels of total magnesium and isocitrate are adjusted to vary the concentrations of the substrate magnesium isocitrate. Since free ADP is the activator for NAD-isocitrate dehydrogenase (Plaut et al., 1974), it is possible that free 5′-APP-NH-P is the inhibitor of the enzyme. The inability to obtain unequivocal results showing competition between substrate and 5′-APP-NH-P may be due to imprecise accounting of the chelated species present as calculated from available stability constants.

Analogues of ADP and ATP in which the bridge oxygen of the pyrophosphoryl group was replaced by a methylene group as in α,β-methylene-ADP (AP-CH_2-P) and in β,γ-methylene-ATP (APP-CH_2-P) were found to be inactive under the conditions tested (Table IV). 5′-Adenosine phosphosulfate (5′-APS), in which the β-phosphate of ADP is replaced by sulfate, was inactive for NAD-isocitrate dehydrogenase (Table IV).

Discussion

Cosubstrate Analogues. An earlier study (Suhadolnik et al., 1977) with four NAD-specific dehydrogenases (horse liver and yeast alcohol dehydrogenases and rabbit muscle gly-

ceraldehyde-3-phosphate and lactate dehydrogenases) showed a differential activity of analogues of NAD^+ with these enzymes. With NFD^+, values of V_{max} were lower with all of the enzymes, particularly for lactate dehydrogenase, even though the K_m of NFD^+ for the latter was about one-fifth of that of NAD^+ (cf. Table II in Suhadolnik et al., 1977; Table I in this work). Modification of the 6-amino group of the purine portion of NAD^+, as in ϵ-NAD^+ and NID^+, led to relatively small changes in the kinetic constants of horse liver alcohol dehydrogenase, but resulted in marked rises of K_m and declines of V_{max} for yeast alcohol dehydrogenase and rabbit muscle glyceraldehyde-3-phosphate dehydrogenase. The bovine heart isocitrate dehydrogenase was inactive with these NAD analogues with concentrations up to 6 mM (Table I); NID^+ does not bind to NAD-isocitrate dehydrogenase since up to 6 mM NID^+ did not inhibit the reduction of NAD^+ (0.2 mM). This suggests that the isocitrate dehydrogenase has a rigid requirement for the 6-amino group on the purine ring of NAD^+

Results with NAD-isocitrate dehydrogenase differed markedly from those with the four enzymes examined previously with NAD^+ analogues modified in the ribosyl group of the adenosine moiety (2′-dNAD and 3′-dNAD) examined for cosubstrate activity. For the alcohol dehydrogenases, glyceraldehyde-3-phosphate dehydrogenase, and lactate dehydrogenase, the values of K_m for the analogues were similar to those of NAD^+, while the values of V_{max} were markedly lower (see Table II in Suhadolnik et al., 1977). With NAD-isocitrate dehydrogenase, the values of K_m for 2′-$dNAD^+$ and 3′-$dNAD^+$ were about five- to sevenfold higher than K_m for NAD^+. While V_{max} was about the same for 2′-$dNAD^+$ and NAD^+, it was substantially lower for 3′-$dNAD^+$ than with NAD^+ (Table I).

X-ray crystallographic studies have shown important similarities in the binding regions of NAD^+ of a number of dehydrogenases (Brändén et al., 1975; Holbrook et al., 1975; Banaszak & Bradshaw, 1975; Wonacott & Biesecker, 1977). Nevertheless, investigations with NAD analogues varying in the adenosine portion have revealed substantial differences in kinetic constants obtained with a number of dehydrogenases (Fawcett & Kaplan, 1962; Pullman et al., 1952; Windmueller & Kaplan, 1961; Lee & Everse, 1973; Suhadolnik et al., 1977). This suggests that differences in the details of the environment in the binding pockets (which are also apparent in the X-ray crystallographic studies) and differences in conformation of cosubstrate analogues (Oppenheimer et al., 1971) have significant effects on the binding of cosubstrates and/or its interaction with substrate. In general, horse liver alcohol dehydrogenase was least affected by cosubstrate modification (see Fawcett & Kaplan, 1962; Suhadolnik et al., 1977), while bovine heart NAD-isocitrate dehydrogenase showed a high degree of specificity. Thus, the nature of substituents at carbon 6 and nitrogen 1 of the adenosine portion of NAD^+ was critical for the isocitrate dehydrogenase (see NID^+ and ϵ-NAD^+ in Table I); in this respect it was similar to rat liver 3-hydroxybutyrate dehydrogenase which was unreactive with NID^+ (Pullman et al., 1952). Furthermore, the isocitrate dehydrogenase had higher values of relative K_m for 2′-$dNAD^+$ and 3′-$dNAD^+$ than other dehydrogenases examined previously (cf. Table II of Suhadolnik et al., 1977, with Table I of this work).

Activity of ADP Analogues. The effects of alterations of functional groups of ADP on allosteric activity for NAD-specific isocitrate dehydrogenases are summarized in Figure 5.

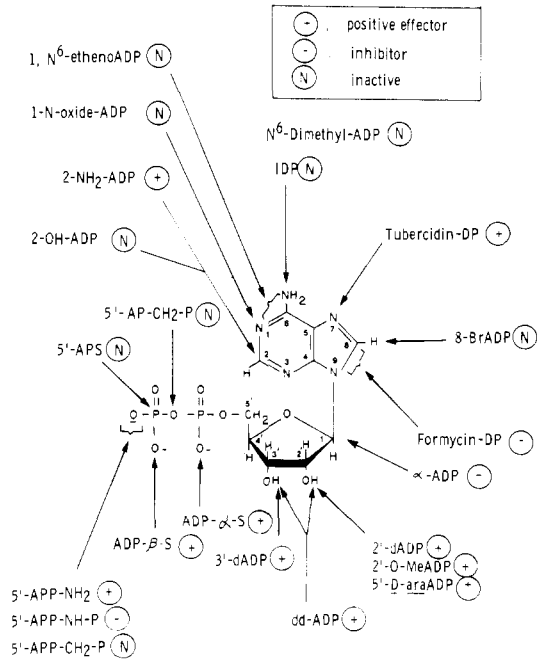

FIGURE 5: Effects of alterations of structure of ADP on effector activity for NAD-specific isocitrate dehydrogenase. The arrows point to the specific location(s) of a functional group(s) in the structure of ADP in which each analogue differs from ADP. The exact nature of the functions varied is described in the text.

There are certain qualitative similarities in structural requirements for binding of NAD^+ and ADP analogues to the cosubstrate and allosteric sites of the isocitrate dehydrogenase. The inosine and 1,N^6-ethenoadenosine derivatives were inactive for either function, the formycin analogues were bound but had opposite effects on enzyme activity, and the 2′-deoxy- and the 3′-deoxyadenosine compounds were active (Table I). However, in contrast to the marked effects of the substituents at carbons 2′ and 3′ on K_m of the NAD analogues (Table I), even extensive modifications at the pentosyl moiety of the ADP analogues resulted in almost complete retention of activity (see 2′-dADP, 3′-dADP, dd-ADP, D-*ara*-ADP, and 2′-*O*-methyl-ADP in Table II). This indicates that, whereas the interaction of the adenosine ribose portion with enzyme is an important feature of coenzyme binding, the ribosyl moiety of ADP must be relatively distant from the polypeptide chains of the enzyme at the allosteric site. The principal function of the ribosyl group of ADP may be as a spacer between the adenine and pyrophosphoryl groups ensuring their proper stereochemical location at the allosteric site. Disruption of the geometric arrangement of the base and pyrophosphate group by cleavage of the bond between carbons 2′ and 3′ of ADP with periodate followed by reduction with $NaBH_4$ (Lerner & Rossi, 1972) led to marked lowering of allosteric activity of this product (1-*O*-[2-*O*-[1(*R*)-(9-adenyl)-2-hydroxyethyl]]glycerol pyrophosphate) for the isocitrate dehydrogenase. The K_m of this cleavage product was 10- to 20-fold larger than that of ADP, and velocity enhancement was only about one-half of that with ADP.[2] Change of the N-glycosidic linkage of ADP from the β to the α configuration has profound effects on the overall geometry of the nucleotide molecule and it is not unexpected that α-ADP is not a positive modifier. Nevertheless, the competitive inhibition by α-ADP of ADP activation (Figure 2) suggests that α-ADP can bind to the allosteric site(s) on the enzyme. Comparison of molecular models of α-ADP and β-ADP suggests that the purine and pyrophosphoryl portions of α-ADP and β-ADP may fit into similar regions. For example, when SRM models of

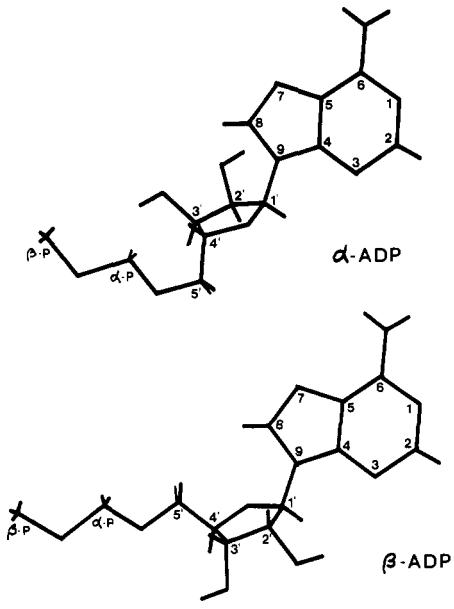

FIGURE 6: Tracings of SRM models of α-ADP and β-ADP. The molecular models have been arranged in the anti conformation as described in the text. The phosphorus and bridge oxygen atoms of the pyrophosphoryl group are shown but the OH groups have been omitted.

α-ADP and β-ADP are arranged in a maximally extended conformation and with the adenine rings superimposed (Figure 6), the furanosyl ring is vertical to the adenine portion in both, carbons 2′ and 3′ and the furanose ring oxygen face in opposite directions in α-ADP and β-ADP, and carbons 5′ of α-ADP and β-ADP are positioned above and below the plane of the furanosyl rings, respectively. Nevertheless, carbons 1′ and 4′ of the diastereoisomers are nearly superimposable and in the same plane with the purine; although the 5′-pyrophosphate groups are not completely superimposable, they can be aligned to fit into the same domain. A number of observations indicated that ADP affects subunit interaction in cardiac NAD-specific isocitrate dehydrogenase (Chen & Plaut, 1963; Giorgio et al., 1970; Shen et al., 1974; Fan et al., 1975). If this were caused by the binding of the purine and pyrophosphoryl portions of ADP to sites on two separate subunits, the ribosyl moiety of ADP would lie in the aqueous medium between the polypeptide chains. With the nucleotide in this location, the interaction of subunits may be relatively unaffected by changes in carbons 2′ and 3′ substituents at the pentosyl group (Table II), but inversion of configuration at the anomeric carbon (i.e., α-ADP) could cause misalignment of subunits leading to loss of allosteric modulation (Figure 2).

The inhibition of ADP activation by FDP (Table III) may be due to the altered alignment of the base of FDP vis-à-vis the pyrophosphoryl group caused by the differences in angles at the carbon–carbon vs. carbon–nitrogen bonds linking the bases to ribose in FDP and ADP, respectively. X-ray crystallography indicates substantial differences in the torsion angles of formycin and adenosine (Koyama et al., 1966) and differences in certain physical and biological properties between formycin and adenosine containing polynucleotides have been attributed on the basis of ORD spectra to the tendency of sequences of formycin residues to assume the syn conformation at the glycosyl linkage (Ward & Reich, 1968). However, other properties may also account for the opposing effects of ADP and FDP at the allosteric site. Thus, the values of pK_a for formycin and adenosine differ (Hori et al., 1964; Robins et al., 1966), the relative concentrations of the amino and imino form tautomers are different for formycin and adenosine (Ceasar & Greene, 1974), and replacement of carbon 8 of the adenine base with nitrogen may alter the interaction of the nucleoside diphosphate with enzyme. It is pertinent in this connection that tubercidin diphosphate (TuDP), which like ADP has a N-glycosidic linkage and a carbon atom at the position corresponding to atom 8, was an effective positive modifier (Table III). Furthermore, TuDP, unlike both ADP and FDP, contains a carbon at the position corresponding to atom 7 of adenine, indicating that a nitrogen at this ring location is not essential for binding of the nucleoside diphosphates to the allosteric site.

Some enzymes which exhibit considerable nucleotide specificity are active with compounds from which the base of the nucleotide has been eliminated. For example, ribose 5-pyrophosphate was a substrate for liver inosine diphosphatase (Heppel et al., 1959) and an inhibitor of RNA polymerase (Sylvester & Dennis, 1977). Ribose 5-pyrophosphate was not an activator for NAD-isocitrate dehydrogenase (Table III).

A free 6-amino group and unsubstituted N-1 of the purine ring of the nucleoside diphosphate are required for modulator activity. Thus, 2-NH_2-ADP (2,6-diaminopurine nucleoside diphosphate) was a positive effector and FDP competitively inhibited the allosteric activation by ADP (Figure 3), whereas 1-*N*-oxide-ADP and ε-ADP (Table III), and, as shown previously (Chen & Plaut, 1963), IDP and GDP, were inactive. N^6,N^6-Dimethylaminopurine nucleoside diphosphate (N^6-dimethyl-ADP) was not an activator, but it was a weak inhibitor of the isocitrate dehydrogenase (Table III). It is uncertain whether it acts at the allosteric site since the large concentrations of this analogue required to test whether the inhibition is competitive with ADP (about 6 mM for 50% inhibition) also result in substantial chelation of magnesium. If the inhibition of N^6-dimethyl-ADP is not competitive with ADP, it may suggest that a specific 6-amino or 6-imino tautomer of ADP is the form of ADP active at the allosteric site. However, steric effects of the N^6-dimethyl substituents interfering with binding of N_6-dimethyl-ADP to enzyme cannot be excluded.

The effects of substituents at positions 2 and 8 of ADP on regulator activity have been tested (Table III). 8-BrADP may be inert because the large bulk of the bromine group prevents access of the analogue to the enzyme binding site. 2-NH_2-ADP was a positive modulator with a 1.5- to 2-fold larger value of K_m than ADP, whereas 2-OH-ADP was neither an activator nor an inhibitor competitive with ADP. The difference in effects of these substituents at the same ring position may be due to the presence of a negatively charged amino acid residue at the enzyme effector binding site located near position 2 of the purine ring of the nucleotide; this could lead to repulsion of the negatively charged 2-hydroxy substituent, but could permit binding when the positively charged 2-amino substituent is present.

Only limited structural modifications could be made in the pyrophosphoryl group of ADP with retention of allosteric activity for bovine heart NAD-isocitrate dehydrogenase. No activity was observed when the bridge oxygen between the α- and β- or β- and γ-phosphate groups of ADP was replaced by a methylene group (AP-CH_2-P or APP-CH_2-P) or when the β-phosphate of ADP was replaced by sulfate as in 5′-APS (Table IV). However, positive modulation of enzyme activity was obtained when the terminal hydroxyl at the β-phosphate group was replaced by an amide (ADP-amide), and replacement of the hydroxyl function by an imidophosphate group (5′-APP-NH-P) gave inhibition competitive with ADP (Table IV and Figure 4A). These results suggest a minimal

requirement for two secondary phosphates of the 5′-pyrophosphoryl group of ADP for interaction at the allosteric site; nevertheless, the primary phosphate seems to be important for maximal effectiveness since the K_m of ADP-amide was about fourfold larger than that of ADP. An arginyl residue has been implicated in positive modulation by ADP of porcine heart NAD-isocitrate dehydrogenase (Hayman & Colman, 1978). Since free ADP is the modifier of the enzyme (Plaut et al., 1974), it seems reasonable that the binding of the pyrophosphoryl group of ADP could occur at an arginyl residue. Furthermore, the lowering of pK_a upon replacement of oxygen by sulfur in the pyrophosphoryl group of ADP (Jaffe & Cohn, 1978) should not interfere with the binding of this group to such a basic residue on the protein. The latter is consistent with the finding that activation of the enzyme by ADP-α-S or ADP-β-S was equivalent to that obtained with ADP (Table IV).

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