Structural Requirements for the Binding of AMP to the Allosteric Site of NAD-Specific Isocitrate Dehydrogenase from Bakers' Yeast

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Received October 6, 1989; Revised Manuscript Received December 14, 1989

ABSTRACT: The specificity of yeast NAD-specific isocitrate dehydrogenase for the structures of the allosteric effector 5'-AMP was examined with analogues modified in the purine ring, pentosyl group, and 5'-phosphate group. An unsubstituted 6-amino group was essential for activation as was the phosphoryl group at the 5'-position. Activity was retained when an oxygen function of the 5'-phosphoryl was replaced by sulfur (Murry & Atkinson, 1968) or by nitrogen (phosphoramidates). 2-NH_2-AMP, 2-azido-AMP, and 8-NH_2-AMP were active; 8-azido-AMP and 8-Br-AMP were inactive. The configuration or nature of substituents about carbons 2' and 3' of the pentosyl portion of AMP was not critical for allosteric activation since AMP analogues containing, e.g., 2',3'-dideoxyribose or the bulky 2',3'-*O*-(2,4,6-trinitrocyclohexadienylidene) substituent (TNP-AMP) were active. TNP-AMP was bound to the enzyme with fluorescence enhancement and had an $S_{0.5}$ for activation similar to the $S_{0.5}$ for AMP. Positive effector activity was decreased when the pentosyl moiety of 5'-AMP was replaced by the six-membered nitrogen-containing morpholine group, indicating that the pentosyl group may be critical as a spacer for the proper geometry of binding to enzyme at the 6-amino and 5'-phosphoryl groups of 5'-AMP. A comparison of molecular models of 5'-AMP with 8,5'-cycloAMP suggests that the species of 5'-AMP required for binding to the enzyme contains the purine and ribose moieties in an anti conformation and positioning of the 5'-phosphate trans with respect to carbon 4'. This is consistent with the finding that (*S*)-8,5'-cycloAMP was a potent negative allosteric modifier (i.e., it increased K_m for isocitrate) whose effect could be reversed competitively by 5'-AMP, whereas the *R* epimer was inactive.

Adenine nucleotides activate NAD-specific isocitrate dehydrogenase from a number of microorganisms (Kornberg & Pricer, 1951; Atkinson et al., 1965; Sanwal et al., 1963) and animal tissues (Chen & Plaut, 1963) but not from plants (Cox & Davies, 1967; Coultate & Dennis, 1969). A characteristic in common for adenine nucleotide activation of the enzyme from different sources is lowering the K_m for isocitrate without affecting V [for review see Plaut (1970)]. A systematic proposal correlating cellular metabolic activity with modulation by varying levels of 5'-AMP of yeast NAD-isocitrate dehydrogenase and certain other enzymes has been developed by Atkinson (1968).

ADP, the activator of the enzyme from animal tissues (Chen & Plaut, 1963), has the additional effects of counteracting the inhibition by NADPH (Gabriel & Plaut, 1984a) and potentiating the activation by magnesium citrate (Gabriel & Plaut, 1984b). The modification by varying concentrations of ADP on the antagonism between NADPH inhibition and citrate activation may contribute to regulating the flux of substrate through the enzyme in mitochondria in different respiratory states (Gabriel et al., 1986).

The specificity of the bovine heart enzyme for ADP activation has been examined with ADP analogues altered with variations in its structural components (Plaut et al., 1979). Several of the ADP analogues either replaced ADP as allosteric activators or were competitive antagonists of ADP. So far, of several nucleoside monophosphates studied (Hathaway & Atkinson, 1963) only adenosine 5'-phosphorothioate (Murray & Atkinson, 1968) was capable of replacing 5'-AMP as an activator of the yeast enzyme. A number of analogues of 5'-AMP, modified in its purine, pentosyl, and phosphate group components, have been found in the present study to bind to the 5'-AMP effector site of yeast NAD-isocitrate dehydrogenase. These findings, combined with changes in spectroscopic properties upon binding to the enzyme of active AMP analogues substituted with a fluorescent group in the pentosyl moiety, should aid in defining further the nature of the AMP binding site. A preliminary report of this work has appeared (Gabriel & Plaut, 1987).

Materials and Methods

Materials. DL-*threo*-Isocitric acid lactone was recrystallized and hydrolyzed as previously described (Gabriel & Plaut, 1980). The free and metal-chelated ligand concentrations are reported on the basis of the DL isomer, even though the enzyme is stereospecific for D-*threo*-isocitrate. 2,6-Diaminopurine nucleoside 5'-monophosphate was prepared by chemical synthesis from the nucleoside (Plaut et al., 1979). NAD^+ and 5'-AMP were purchased from Boehringer-Mannheim (Indianapolis, IN), Sephadex G-25-SF and 2',3'-dideoxy-AMP[1]

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[1] Abbreviations: TNP-AMP, 2',3'-*O*-(2,4,6-trinitrocyclohexadienylidene)adenosine 5'-monophosphate; 2-AZPET-AMP, 2-[[2-(*p*-azidophenyl)ethyl]thio]adenosine 5'-monophosphate; dansyl-AMP, adenosine 5'-*N*-[*β*-(dansylamino)ethyl]phosphoramidate; 8,5'-cycloAMP, 8,5'-cycloadenosine 5'-phosphate; AMP-anisidate, adenosine 5'-phosphoro-*p*-anisidate; methylmorpholine-AMP, 6-adenyl-4-methyl-2-(hydroxymethyl)morpholine phosphate; (amino-*n*-butyl)morpholine-AMP, 6-adenyl-4-(4-aminobutyl)-2-(hydroxymethyl)morpholine phosphate; *lin*-benzoadenine, 8-aminoimidazo[4,5-*g*]quinazoline; *lin*-benzo-AMP, *lin*-benzoadenosine 5'-monophosphate; *ara*-AMP, adenine 9-*β*-D-arabinofuranoside 5'-monophosphate; 2-NH_2-AMP, 2,6-diaminopurine nucleoside 5'-monophosphate; 8-NH_2-AMP, 8-aminoadenosine 5'-monophosphate; 8-Br-AMP, 8-bromoadenosine 5'-monophosphate; 3'-deoxy-AMP, 3'-deoxyadenosine 5'-monophosphate; 2'-deoxy-AMP, 2'-deoxyadenosine 5'-monophosphate; 2',3'-dideoxy-AMP, 2',3'-dideoxyadenosine 5'-monophosphate; DTT, dithiothreitol.

0006-2960/90/0429-3528$02.50/0

were from Pharmacia (Piscataway, NJ), alkaline phosphatase, type III from *Escherichia coli*, was from Sigma (St. Louis, MO), and bakers' yeast was from Red Star (Willow Grove, PA).

TNP-AMP from Molecular Probes (Eugene, OR) was purified by chromatography on Whatman 3 MM paper with 1-butanol/acetic acid/H_2O (40:10:25) as the mobile phase. The final concentration of TNP-AMP was determined spectroscopically at 408 nm in 0.2 M Tris-HCl at pH 8.0; $\epsilon = 26.0 \times 10^3$ M^{-1} cm^{-1} (Hiratsuka, 1982).

Adenosine 5′-phosphoro-*p*-anisidate was synthesized by the method of Moffatt and Khorana (1961). The corresponding fluorescent adenosine 5′-*N*-[β-(dansylamino)ethyl]phosphoramidate was prepared by an adaptation of the method of Moffat and Khorana (1961) using β-(dansylamino)ethylamine and 5′-AMP as the starting materials. 6-Adenyl-4-methyl-2-(hydroxymethyl)morpholine phosphate and the corresponding 4-(4-aminobutyl)morpholine-AMP analogue were synthesized by the method of Kym (1963). *lin*-BenzoAMP was the gift of Dr. N. J. Leonard, Department of Chemistry, University of Illinois; 2-AZPET-AMP (Cristalli & Mills, 1987) was the gift of Dr. D. C. B. Mills, Temple University School of Medicine; 2-azido-5′-AMP was the gift of Dr. B. Haley, University of Kentucky; *ara*-A 5′-monophosphate, tubercidin 5′-monophosphate, and toyocamcin 5′-monophosphate were gifts of Dr. R. J. Suhadolnik of Temple University School of Medicine; the *R* and *S* epimers of 8,5′-cycloadenosine were the gift of Dr. J. A. Raleigh, North Carolina Memorial Hospital, Chapel Hill, NC. All other nucleoside phosphates were purchased from Sigma. Other chemicals were reagent grade and were obtained commercially.

Determination of Purity of Nucleotides. The solvent systems used for development of thin-layer chromatograms were as follows: I, 2-propanol/NH_4OH/H_2O (7:1:2); II, 95% ethanol/1 M ammonium acetate (1:1); III, isobutyric acid/1 M NH_4OH/0.1 M EDTA (250:150:4); IV, ethanol/2 M LiCl (1:1) saturated with boric acid (Schwartz & Drack, 1975). Eastman Chromagram No. 13255 (cellulose) was the stationary phase for systems I–III and Macheray-Nagel Polygram CEL300 PEI for system IV. Nucleotides were detected under ultraviolet light. The purity of nucleotides, especially the AMP content, was also examined by HPLC using a Waters C-18 Microbondapak reversed-phase column (3.9 mm × 30 cm) with a mobile phase of 0.1 M $(NH_4)_2HPO_4$ at pH 5.0 at a flow rate of 3 mL/min. Column effluents were monitored at 254 nm.

Examination of Nucleotides for Contamination by 5′-AMP. Since the enzyme has a K_m for activation by 5′-AMP in the micromolar range, it was important to be certain that the effector activity of other nucleotides could not be attributed to contamination by AMP. The chromatographic procedures used to monitor the purity of nucleotides were capable of detecting an AMP content in excess of 0.1%. The migration constants of nucleotides in TLC and HPLC systems are given below. By use of reversed-phase HPLC, the retention times (shown in parentheses) were as follows: 5′-AMP (7.6 min), 2-NH_2-AMP (5.0 min), adenosine 3′,5′-bisphosphate (4.3 min), adenosine 2′,5′-bisphosphate (4.9 min), tubercidin MP (4.3 min), adenosine 5′-phosphoramidate (9.2 min), 8-NH_2-AMP (11.7 min), 3′-deoxy-AMP (13.2 min), 2′-deoxy-AMP (15.1 min), toyocamycin MP (31.4 min), 6-adenyl-4-(4-aminobutyl)-2-(hydroxymethyl)morpholine phosphate (17.9 min), 6-adenyl-4-methyl-2-(hydroxymethyl)morpholine phosphate (23.5 min), 2′,3′-dideoxy-AMP (35.2 min), and formycin MP (> 65 min). TNP-AMP (7.6 min) and *ara*-AMP (8.3 min) could not be distinguished from AMP in this HPLC system, but they were separable by TLC in system IV. Other R_f values are shown in parentheses for the nucleotides cited: (system I) AMP (0.08), AMP-anisidate (0.44), adenosine 5′-phosphoramidate (0.25), 6-adenyl-4-methyl-2-(hydroxymethyl)morpholine phosphate (0.35); (system II) AMP (0.38), 2-azido-AMP (0.00);[2] (system III) AMP (0.62), 6-adenyl-4-(4-aminobutyl)-2-(hydroxymethyl)morpholine phosphate (0.82); (system IV) AMP (0.20), *ara*-AMP (0.49), TNP-AMP (0.03), adenosine 5′-*N*-[β-(dansylamino)ethyl]-phosphoramidate (0.83).

Purification of NAD+. Commercial NAD^+ contains about 0.3% 5′-AMP as determined by the aforementioned HPLC system (retention times for AMP and NAD^+ were 9.5 and 24 min, respectively). At a K_m of 5 μM, this amount of contamination by AMP is large enough to partially activate the enzyme at higher concentrations of NAD^+. Attempts to free NAD^+ from AMP by published procedures using ion-exchange chromatography on Dowex 1 or DEAE-cellulose were not successful since significant formation of AMP from NAD^+ occurred during the chromatographic process.

NAD^+ was purified by incubating 0.5 g of NAD^+ in 5 mL of 50 mM Tris–acetate at pH 7.4 with 3 units of alkaline phosphatase for 1 h, followed by HPLC on a 5-μm Altex Ultrasphere ODS C18 column (10 mm × 25 cm) with 0.1 M formic acid (pH 2.4) as the mobile phase at a flow rate at 5.5 mL/min. The NAD^+-containing fractions were brought to dryness by lyophilization. The AMP content of the purified NAD^+ was less than 0.04%.

Enzyme Purification. The standard assay for monitoring enzyme purification contained 0.1 M Tris–acetate at pH 7.6, 2 mM NAD^+, 1 mM AMP, 4 mM magnesium acetate, and 2 mM DL-isocitrate. Protein concentration was determined by the method of Bradford (1976). One unit of enzyme activity was defined as the amount of enzyme required to catalyze the production of 1 μmol of NADH/min at 25 °C. All purification procedures were carried out at 4 °C unless stated otherwise.

(*1*) *Extraction.* Fresh bakers' yeast cake (22.5 kg) was suspended in 7.5 L of a buffer at pH 7.0 containing 0.4 M KCl, 20 mM potassium phosphate, 0.1 mM DTT, and 1 mM phenylmethanesulfonyl fluoride. Cells were broken with an Impandex glass bead grinding mill. Protamine sulfate was added to a final concentration of 0.33%. The mixture was left to stand for 1 h and then centrifuged at 11000*g* for 60 min.

(*2*) *Ammonium Sulfate Fractionation.* The turbid supernatant fluid was clarified by filtration through a 10 cm × 27.5 cm column containing a mixture of equal volumes of phosphocellulose and Sephadex G-25 which had been equilibrated with a buffer at pH 7.0 containing 0.4 M KCl, 20 mM potassium phosphate, 0.1 mM DTT, and 0.01% sodium azide. One column volume of extract was applied to the column, followed by 1 column volume of equilibration buffer. The effluents were combined and used in the subsequent step. The column was regenerated with 2 column volumes of 0.1 M potassium phosphate–1.5 M KCl–0.01% sodium azide at pH 7.0, followed by 1 column volume of equilibration buffer.

The filtrates were combined, brought to 55% saturation by the addition of 333 g of ammonium sulfate/L of solution, and allowed to stand for about 18 h. After centrifugation, the pellet containing the activity was dissolved in a minimal volume of a buffer at pH 7.6 containing 30 mM sodium phosphate, 0.1

[2] After 5-min exposure to UV light on the cellulosic medium before chromatography; R_f = 0.40 before UV irradiation.

Table I: Purification of NAD-Isocitrate Dehydrogenase from Bakers' Yeast[a]

step no.	fraction	volume (mL)	protein (mg/mL)	activity (units/mL)	activity (units/mg)	yield (%)	purification (x-fold)
1	yeast extract	10000	31.5	2.4	0.075		1
2	ammonium sulfate precipitation	3000	80.0	11.3	0.14	100	2
3	hydroxylapatite	640	25.5	30.7	1.2	58	16
4	Blue Sepharose	241	2.0	40.0	20	28	270
5	Sephadex G-200	125	1.25	48.0	38	18	510

[a] Yeast cake (22.5 kg) was used as the starting material.

mM DTT, and 1 mM phenylmethanesulfonyl fluoride. Activity was fully retained for 1 year when this preparation was stored at −90 °C.

(*3*) *Hydroxylapatite Chromatography.* A quantity of the active fraction from step 2 containing no more than 3 g of protein was dialyzed against a buffer at pH 6.5 containing 10 mM potassium phosphate and 0.1 mM DTT. It was applied to a 4 cm × 17 cm column of hydroxylapatite equilibrated with the same buffer. The column was washed with equilibration buffer followed by a solution containing 0.1 M potassium phosphate and 0.1 mM DTT at pH 6.5 until the absorbance at 280 nm of effluents was negligible. Enzyme was eluted with 0.2 M potassium phosphate–0.1 mM DTT at pH 6.5. Fractions containing the activity were concentrated in Amicon filter cones (CF 25) and buffer exchanged against the same buffer used to equilibrate the Sepharose Blue column in step 4. The concentrated fraction can be stored at −90 °C following the addition of 20% (v/v) glycerol.

(*4*) *Blue Sepharose Chromatography.* This chromatographic step was done at 25 °C. An aliquot from the previous step containing about 200 enzyme units was applied to a 0.7 cm × 15 cm column of Sepharose Blue CL-6B equilibrated with a buffer at pH 7.5 containing 20 mM Tris-HCl, 1 mM sodium citrate, 4 mM magnesium sulfate, and 0.2 mM DTT. The column was washed with the equilibration buffer followed by an overnight wash with a buffer at pH 7.5 containing 20 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl, and 0.1 mM DTT at a flow rate of 0.1 mL/min. The enzyme was eluted with the same buffer by using a linear gradient between 100 and 300 mM NaCl in a total volume of 100 mL. The enzyme was eluted by about 140 mM NaCl. The fractions containing activity were pooled and concentrated at 4 °C in a collodion bag (mol wt cutoff 75000) and buffer exchanged into the same buffer used in step 5.

(*5*) *Sephadex G-200 Chromatography.* About 200 units of the fraction from step 4 was applied to a 1.6 cm × 60 cm column of Sephadex G-200 equilibrated with a buffer at pH 7.5 containing 0.1 M Tris-HCl, 0.3 M NaCl, and 0.1 mM DTT. The column was eluted with the same buffer. Fractions containing activity were concentrated and stored at −90 °C in a buffer at pH 7.6 containing 30 mM potassium phosphate, 0.1 mM DTT, and 20% (v/v) glycerol.

The results of a typical purification shown in Table I have been normalized with respect to yield. In practice, aliquots of the ammonium sulfate precipitate fraction from step 2 were processed through the rest of the purification as required. The average recovery of enzyme activity from yeast extract was 20% with a 500-fold purification. The specific activity of the purified enzyme ranged from 31 to 47 units/mg. In SDS–polyacrylamide gel electrophoresis of the purified enzyme, 80% of the protein was recovered in two closely spaced bands of about equal intensity. Their location on the gel corresponded to a subunit molecular weight of about 40000, in agreement with Barnes et al. (1971).

Kinetics. All assays were initiated by the addition of purified enzyme to a final concentration of 2×10^{-4} mg/mL. When necessary, the enzyme was diluted into a buffer containing 50 mM Tris–acetate at pH 7.4, 1 mg/mL bovine serum albumin, 1 mM 1,3-dimercaptopropanol, and 20% (v/v) glycerol. Initial velocities were measured in the presence of 50 mM Tris–acetate at pH 7.4 with 1 mg/mL bovine serum albumin and were expressed as units per milligram of protein. One unit of enzyme activity was defined as the amount of enzyme required to catalyze the production of 1 μmol of NADH/min at 25 °C. The concentrations of free and metal-chelated forms of isocitrate, NAD^+, and AMP were maintained at the levels indicated in the text and figures by adjusting the total ligand and magnesium concentrations as described previously (Gabriel et al., 1986). Kinetic parameters were calculated from initial velocities by fitting the data to the appropriate computer programs developed by Cleland (1963).

Fluorescence Spectroscopy. Fluorescence spectra were measured in a 0.1-mL, 0.32-cm path length cell by using a Perkin Elmer Model 650-10S fluorimeter. All measurements were corrected for fluctuations in the exciting lamp intensity. Fluorescent intensities were corrected for inner-filter effects by using (Lakowicz, 1983)

$$F_{cor} = F_{obs} \text{ antilog } [(Abs_{ex} + Abs_{em})/2] \quad (1)$$

The fluorescent titration experiments (λ_{ex} = 410 nm, λ_{em} = 535 nm) were carried out by keeping the concentration of TNP-AMP constant while decreasing the enzyme concentration by dilution with buffer containing the same initial analogue concentration. The dissociation constant, K_d, was determined by fitting the data to (Scheffer & Fromm, 1986)

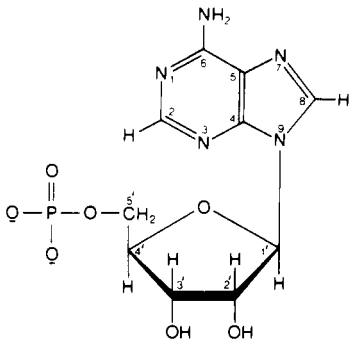
$$1/f = \left(\frac{n}{K_d}\right)\left(\frac{E_T}{b}\right) - \frac{L_T}{K_d} \quad (2)$$

where b and f are the fraction of bound and free ligand, respectively. Enzyme samples were buffer exchanged into 50 mM Tris–acetate at pH 7.4 immediately before the fluorescence measurement by passage through a column (0.2 cm × 8 cm) of Sephadex G-25 SF.

Results

In agreement with Hathaway and Atkinson (1963), 5′-AMP lowered the apparent $S_{0.5}$ for the substrate, free isocitrate (Gabriel & Plaut, 1986), without changing V or the Hill number for isocitrate. In the present studies, the reaction mixtures at pH 7.4 contained acetate as the only counterion to minimize the inhibitory effects of other anions reported by Cennamo et al. (1967) and enzyme protein was kept above 2×10^{-4} mg/mL (Gabriel & Plaut, 1986). Under these conditions and at subsaturating concentrations of NAD^+ and Mg^{2+}, 0.20 mM AMP lowered the apparent $S_{0.5}$ for free DL-isocitrate by 16-fold from 1.4 ± 0.07 mM (47 data points) to 0.09 ± 0.004 mM (41 data points) (Figure 1) without a significant change in the Hill number (n) for isocitrate (n = 2.4 vs 1.9 without and with AMP, respectively). At a constant concentration of free DL-isocitrate (0.15 mM) the velocity response to activation by varied AMP was hyperbolic with an apparent $S_{0.5}$ for AMP of 4.9 ± 0.5 μM (11 data points) (inset

Table II: Effectiveness of AMP Analogues[a]



analogue	v (units/mg)	v/v_0(no AMP) (ratio)	position(s) modified
no addition[b]	1.46 ± 0.6 (31)	1.0	
(*S*)-8,5′-cycloAMP[c]	0.0 ± 0.0 (2)	0.0	8, 5′
(amino-*n*-butyl)morpholine-AMP	3.7 ± 0.4 (4)	2.5	ribosyl ring expansion
AMP-anisidate	4.7 ± 0.6 (5)	3.2	5′-P
dansyl-AMP	5.4 ± 0.3 (3)	3.7	5′-P
formycin MP	6.0 ± 0.5 (5)	4.1	8, 9
methylmorpholine-AMP	6.2 ± 0.2 (2)	4.3	ribosyl ring expansion
adenosine 5-monophosphoramidate	10.7 ± 1.0 (3)	7.3	5′-P
2′,3′-dideoxy-AMP	12.3 ± 0.3 (2)	8.4	2′, 3′
2-azido-AMP	13.6 ± 0.1 (2)	9.3	2
2-AZPET-AMP	14.1 ± 0.1 (2)	9.6	2
TNP-AMP	14.4 ± 0.6 (3)	9.9	2′, 3′
ara-AMP	15.8 ± 0.8 (6)	10.8	2′
3′-deoxy-AMP	16.0 ± 0.4 (3)	11.0	3′
2′-deoxy-AMP	15.6 ± 0.1 (3)	10.7	2′
adenosine 3′,5′-bisphosphate	16.1 ± 0.3 (3)	11.0	3′
8-NH_2-AMP	17.7 ± 0.3 (5)	11.8	8
tubercidin MP	18.7 ± 0.7 (3)	12.8	7
toyocamycin MP	19.0 ± 0.4 (2)	13.0	7
2-NH_2-AMP	20.3 ± 0.6 (3)	13.9	2
5′-AMP	19.6 ± 0.8 (11)	13.4	

[a] Assay conditions: Free Mg^{2+} (0.15 mM), BSA (1 mg/mL), NAD^+ (0.33 mM), free DL-isocitrate (0.5 mM), Tris–acetate (50 mM, pH 7.4), enzyme (2×10^{-4} mg/mL), and nucleotides (0.2 mM) as indicated. Note that v and v_0 are velocities in the presence and absence of analogue, respectively. [b] The following analogues were ineffective in replacing 5′-AMP as an activator of the enzyme: 5′-GMP, 5′-UMP, 5′-CMP, 5′-IMP, 3′,5′-cAMP, 2′-AMP, 3′-AMP, 1,N^6-ϵAMP, adenosine, adenosine + PO_4, adenosine 5′-monosulfate, 8-Br-AMP, 8-azido-AMP, N^6-methyladenosine 5′-monophosphate, 2′,5′-ADP, 5′-αAMP, *lin*-benzoadenosine MP, adenosine 5′-carboxylic acid. [c] The (*R*)-8,5′-cycloAMP analogue had no effect on enzyme activity.

of Figure 1), suggesting a single type of enzyme binding site.

Nucleotides varying in the phosphate group, the pentosyl moiety, and the base portion were tested to define the structural requirements for 5′-AMP activation. Their kinetic effects were compared to those with and without 5′-AMP in an assay medium containing subsaturating substrate and Mg^{2+} concentrations as described in Table II.

Variations of Nucleoside 5′-Phosphate Bases. The ability to activate the enzyme was lost when the purine portion of 5′-AMP was replaced by a pyrimidine (5′-UMP and 5′-CMP) or when the free 6-amino substituent on the purine ring was absent as in 5′-GMP, 5′-IMP, and 1,N^6-ϵAMP. *lin*-Benzoadenosine nucleotides were capable of replacing adenylates in a number of enzyme-catalyzed reactions (Leonard et al., 1978). However, *lin*-benzo-AMP was ineffective as an activator for the isocitrate dehydrogenase, perhaps because the 6-amino group is shifted out of position as a result of the purine ring expansion.

Replacement of the hydrogen at position 8 of AMP by a bromo or azido group resulted in the loss of effector activity. This effect may be due to the size or electronegativity of these substituents since the degree of activation by 8-NH_2-AMP was nearly the same as that of AMP. Furthermore, a mechanism involving a proton loss at position 8 does not appear to have a role in the binding of AMP to the enzyme since an exchange of the 8-hydrogen with deuterium could not be detected by NMR spectroscopy upon incubating AMP in a ^2H_2O containing medium for 24 h in the presence of the enzyme.

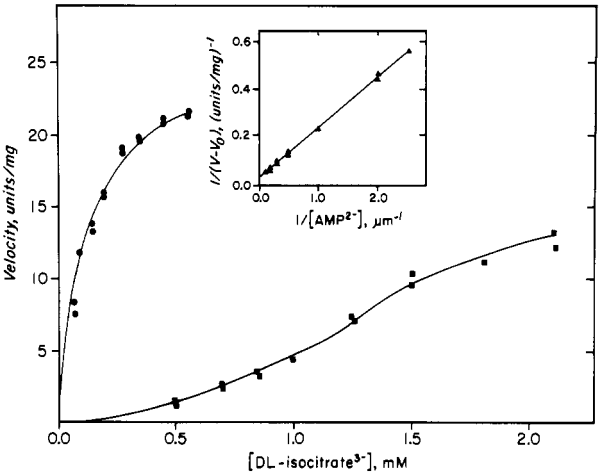


FIGURE 1: Effect of AMP on the apparent $S_{0.5}$ for isocitrate. Enzyme activity was measured without AMP (■) and in the presence of 0.2 mM AMP^{2-} (●). The reaction mixture at pH 7.4 contained 0.33 mM NAD^+, 0.15 mM free magnesium, 1 mg/mL BSA, and 2×10^{-4} mg/mL yeast enzyme. Inset: Double-reciprocal plot of $v - v_0$ vs the concentration of AMP^{2-} at a constant concentration of 0.5 mM $isocitrate^{3-}$. Note that v_0 is velocity in the absence of AMP.

Activation comparable to that of AMP was obtained with 5′-AMP derivatives substituted at carbon 2 (2-NH_2-AMP, 2-azido-AMP, and 2-AZPET-AMP) or by replacement of the

nitrogen at position 7 by carbon as in tubercidin MP or toyocamycin MP. However, the replacement of the imidazole portion of AMP by a pyrazole ring in formycin MP reduced activation to about one-third at equivalent concentrations.

Variations in the Pentosyl Group. Even extensive modification at the pentosyl moiety of 5′-AMP resulted in substantial retention of activity (see *ara*-AMP, 2′-deoxy-AMP, 3′-deoxy-AMP, and 2′,3′-dideoxy-AMP). However, whereas adenosine 3′,5′-bisphosphate was nearly comparable to 5′-AMP as an activator, adenosine 2′,5′-bisphosphate and 3′,5′-cAMP were inactive. ATP derivatized with a trinitrophenyl group at the 2′- and/or 3′-positions (TNP-ATP) replaced ATP as a substrate for myosin ATPase and adenylate kinase (Hiratsuka, 1982). The fluorescence analogue TNP-AMP was an excellent activator for NAD-isocitrate dehydrogenase.

Expansion of the pentosyl moiety of 5′-AMP to a six-membered nitrogen-containing ring by the method of Kym (1963) yielded the corresponding morpholine derivatives. The methylmorpholine and (amino-*n*-butyl)morpholine analogues activated the enzyme but to a lesser extent than AMP. The values of $S_{0.5}$ for activation by the methylmorpholine and (amino-*n*-butyl)morpholine derivatives were 0.15 ± 0.01 mM (14 data points) and 0.19 ± 0.04 mM (12 data points), respectively, values 30- to 40-fold higher than the apparent $S_{0.5}$ for AMP. Furthermore, since the maximum velocities for activation by the methyl- and (amino-*n*-butyl)morpholine derivatives of 9.5 and 5.9 units/mg, respectively, were less than the value for AMP, it appears that the enzyme conformational change that they induce is less favorable than that of AMP for catalysis.

Modification of the 5′-Phosphoryl Group. Murray and Atkinson (1968) demonstrated that replacement of the hydroxyl by a sulfhydryl group at the 5′-phosphate group of AMP resulted in retention of positive effector activity for yeast NAD-isocitrate dehydrogenase. As with adenosine 5′-phosphorothioate, substitution of one hydroxyl at the phosphate of 5′-AMP by a nitrogen in amide linkage yielded compounds which were activators. Thus, at comparable concentrations, adenosine 5′-monophosphoramidate was about half as effective as 5′-AMP. Effector activity could also be demonstrated for phosphoramidates with bulkier nitrogen-containing groups, such as the anisidate and [β-(dansylamino)ethyl]phosphoramidate derivatives, although they were only about one-fourth as effective as activators as AMP. However, the inner ester adenosine 3′,5′-cyclic monophosphate was inactive.

There are two stereoisomers of 8,5′-cycloAMP depending on the configuration of the phosphate group at carbon 5′ (Hampton et al., 1972). The *R* isomer of 8,5′-cycloAMP was inert (Table II). However, when assayed in the absence of AMP, with 0.33 mM NAD^+, 0.15 mM free magnesium, and 1.4 mM DL-isocitrate, the *S* form was a potent inhibitor of the enzyme with $I_{0.5}$ of 0.4 μM as compared to $S_{0.5}$ of 5 μM for activation of AMP. In separate experiments, the inhibition by (*S*)-8,5′-cycloAMP was shown to be competitive with respect to both AMP and isocitrate, increasing the apparent $S_{0.5}$ for DL-isocitrate, without affecting *V* or the Hill number (*n*) for isocitrate (data not shown).

The Fluorescent Probe TNP-AMP. In the absence of enzyme a 22-nm blue shift from 546 to 524 nm in the emission spectrum of TNP-AMP was observed as the fraction of dimethylformamide in the solvent was increased. Accompanying the blue shift was a marked increase, by a factor of 175, in the fluorescent intensity of the analogue. A similar blue shift (22 nm) and enhancement (40-fold) were observed when 50 mM Tris–acetate at pH 7.4 replaced water in the solvent (not shown). The lower enhancement observed at pH 7.4 reflects the observation by Hiratsuka (1982) that only the neutral or basic form of the analogue is the fluorescent species, resulting in an increase in the intensity of the analogue when measured in buffer compared to a solvent containing only water.

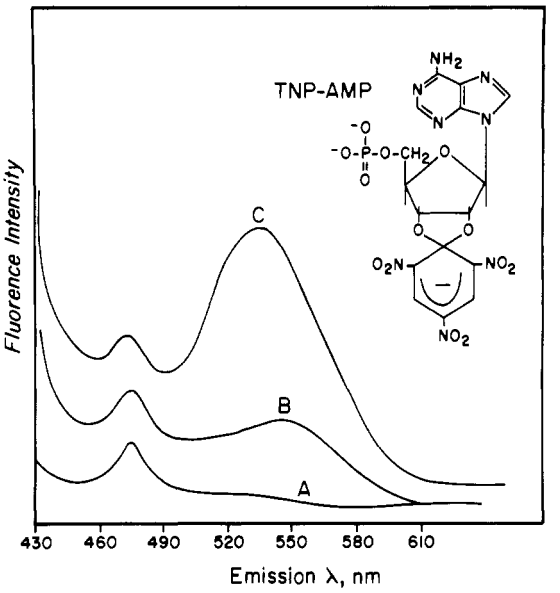


FIGURE 2: Fluorescence emission spectra of TNP-AMP in the presence and absence of yeast isocitrate dehydrogenase. Curve A: 50 mM Tris–acetate at pH 7.4, i.e., buffer. Curve B: 10.5 μM TNP-AMP in buffer. Curve C: 10.5 μM TNP-AMP and 1.21 μM enzyme (octamer) in buffer. The excitation wavelength was 410 nm.

When the fluorescence of the analogue was measured in the presence of yeast NAD-specific isocitrate dehydrogenase, the intensity of TNP-AMP was enhanced only 4-fold while the wavelength of maximum emission shifted only 8 nm, from 543 to 535 nm (Figure 2). The marked changes in the fluorescence properties of the analogue, i.e., a 22-nm vs an 8-nm blue shift and a 40–175-fold enhancement vs a 4-fold enhancement in the absence and presence of enzyme, respectively, suggest that the trinitrophenyl group is not in a very hydrophobic environment when the analogue is bound to the enzyme (Figure 2). The fluorescent intensity of TNP-AMP was not affected by the further addition of magnesium (0.2 mM) and isocitrate (1.0 mM). However, the further addition of 5′-AMP (0.2 mM) decreased the fluorescent intensity to the value in the absence of enzyme, indicating that the analogue and AMP compete for the same site on the enzyme (data not shown). Identical results were obtained regardless of whether or not metal and isocitrate were present, suggesting that these ligands were not required for the binding of TNP-AMP to the enzyme. The small enzyme-induced fluorescent enhancement of TNP-AMP was sufficient to allow for the spectroscopic determination of the binding of the analogue to the enzyme according to the procedure of Scheffer and Fromm (1986). In the absence of metal, TNP-AMP bound to the enzyme with K_d = 4.9 ± 1.4 μM (10 data points) (Figure 3) and remained statistically the same when metal was added (data not shown).

The Dansyl-AMP Analogue. The fluorescent analogue adenosine 5′-*N*-[β-(dansylamino)ethyl]phosphoramidate, a relatively poor activator (Table II), binds to the enzyme with an apparent $S_{0.5}$ of 29.2 ± 1.8 μM (15 data points), close to the value observed for AMP (Figure 1). The fluorescence of the analogue was examined in solvents of decreasing polarity, using an excitation wavelength of 340 nm. In a manner similar to the TNP-AMP analogue, a 44-nm blue shift and a 25-fold fluorescence enhancement (at 500 nm) was observed. How-

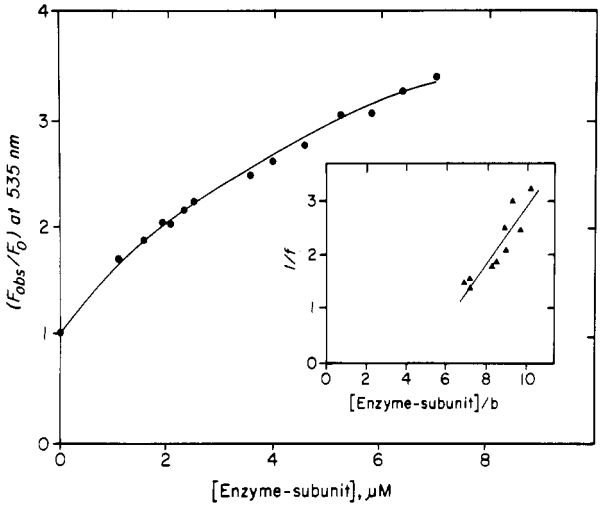


FIGURE 3: Ligand binding study of TNP-AMP (10.5 μM) to yeast isocitrate dehydrogenase. The buffer used was 50 mM Tris–acetate at pH 7.4 while the excitation wavelength was 410 nm. F_0 and F_{obs} are the fluorescence intensities in the absence and presence of protein, respectively, corrected for inner-filter effects. Inset: Determination of the dissociation constant, K_d, by using eq 2, where f and b are the fractions of free and bound ligand, respectively.

ever, in contrast to TNP-AMP, no change in fluorescence emission or polarization of the analogue was observed when bound to the enzyme (not shown), suggesting that the 5′-dansyl group was free to rotate in a nonhydrophobic environment presumably exposed to the solvent.

Discussion

Structural features of 5′-AMP that appear to be absolutely required for effector activity by yeast NAD-isocitrate dehydrogenase are a free *exo*-amino group at carbon 6 and a phosphate group at position 5′. The structural specificity for the 6-amino substituent on the purine ring was very narrow since it could not be replaced by the corresponding 6-(monomethylamino) group. The structural requirement for the 5′-phosphate group was less demanding since effector activity was retained upon replacement of one of the phosphate hydroxyls as in the nitrogen-containing 5′-phosphoramidate, 5′-anisidate, etc.; however, the internal ester 3′,5′-cyclic AMP was inactive.

The configuration or nature of substituents about carbons 2′ and 3′ of the pentosyl portion of AMP is not critical for allosteric activation since AMP analogues containing 2′-deoxyribose, 3′-deoxyribose, 2′,3′-dideoxyribose, D-arabinose, and modification at the 2′- and/or 3′-hydroxyl group with the bulky trinitrophenyl substituent retained allosteric activity (Table II). However, while 3′,5′-ADP was a good activator, 2′,5′-ADP had no effect. Molecular models indicate possible ionic interaction between the 2′-phosphate (but not 3′-phosphate) and nitrogen 3, resulting in substantial changes in the geometric location of the purine and ribosyl portions of 2′,5′-ADP, thus preventing binding to the enzyme. It appears that, in the binding of AMP to the enzyme, the 2′- to 3′-carbon portion may be exposed to a rather large solvent region within the protein. This would be consistent with the lack of specificity of this region of the molecule for allosteric activation (Table II) and the relatively small fluorescence enhancement and blue shift of TNP-AMP on binding to the enzyme when compared to these changes in fluorescence properties of TNP-AMP in solvents of decreasing polarity (Figure 2). The principal function of the ribosyl group of 5′-AMP may be as a spacer between the adenine and the phosphoryl groups, ensuring their proper stereochemical location at the allosteric site. Alteration of the geometric arrangement between the base and the phosphoryl group by expanding the pentosyl portion of AMP to the corresponding six-membered nitrogen-containing morpholine derivatives led to a marked decrease in allosteric activity of these products which had a higher $S_{0.5}$ and lower V for activation than 5′-AMP (Table II).

If the spacing of the purine moiety and that of the 5′-phosphate group are important for enzyme activation, it seems likely that the glycosidic bond angle, χ_{CN}, and puckering of the ribosyl portion of the nucleotide affect activity. The natural activator 5′-AMP has the anti configuration (Sundaralingam, 1969). It is possible that 8-Br-AMP was inactive and formycin monophosphate was a mediocre activator (Table II) because they exist in the syn conformation (Sarma et al., 1974; Giranda et al., 1988). However, other explanations are possible. Thus, the inactivity of 8-Br-AMP and 8-azido-AMP (Table II) may be due to the partial negative charge of the bromo and azido substituents (Cartwright et al., 1976) since the positively charged 8-NH_2-AMP is a good activator (Table II). On the other hand, the rotation about the glycosidic bond in formycin is less hindered than in adenosine (Ward & Reich, 1968; Haschemeyer & Rich, 1967. Thus, limited conversion of the syn to the presumably more favorable (for binding) anti conformation of formycin phosphate may explain why this nucleotide is an, albeit weak, activator.

The enzyme effector activity of 8,5′-cycloadenosine 5′-monophosphate was examined since the covalent linkage between carbons 8 and 5′ should place the compound unequivocally into the anti conformation. The compound was synthesized as an epimeric mixture by Hampton, Harper and Sasaki (Hampton et al., 1972). The absolute configuration of the two possible $C_{5'}$ epimers of 8,5′-cycloadenosine 5′-monophosphate was established by NMR spectroscopy (Raleigh & Blackburn, 1978; Raleigh & Fuciarelli, 1985) and by X-ray crystallography of the corresponding 8,5′-cycloadenosine monohydrate (Haromy et al., 1980). The substrate specificity of the epimers has been tested with a limited number of enzymes (snake venom 5′-nucleotidase, porcine adenylate kinase), and only the R epimer of 8,5′-cycloAMP was found to be active (Raleigh & Kremers, 1975; Raleigh et al., 1976; Raleigh & Blackburn, 1978). The R epimer of 8,5′-cycloAMP was inactive for yeast NAD-isocitrate dehydrogenase, whereas the S epimer was a potent negative effector for the enzyme (Table II). Under equivalent incubation conditions, (*S*)-8,5′-cycloAMP bound an order of magnitude more tightly to the enzyme ($I_{0.5}$ = 0.4 μM) than AMP ($S_{0.5}$ = 5 μM). Kinetic evidence indicates that (*S*)-8,5′-cycloAMP and AMP bind to the same regulator site on the enzyme. Thus, the inhibition by (*S*)-8,5′-cycloAMP was reversed competitively by AMP, and in contrast to the positive effector AMP which lowered $S_{0.5}$ for isocitrate, the negative modifier (*S*)-8,5′-cycloAMP raised $S_{0.5}$ for isocitrate. However, neither AMP nor (*S*)-8,5′-cycloAMP affected V or the Hill number for isocitrate, and as expected, the inhibition by (*S*)-8,5′-cycloAMP was reversed competitively by isocitrate.

Computer-generated structures were developed from available X-ray crystal data for 5′-AMP and the two AMP analogues (*S*)-8,5′-cycloAMP and 3′,5′-cAMP to correlate structural features with their ability to modulate the activity of yeast NAD-isocitrate dehydrogenase. In the representation of the structures in Figure 4 all three AMP analogues were compared in the anti conformation, and the three molecules were superimposed at the adenine ring moieties by a least-squares procedure to minimize the distances between atoms N_1, N_7, and N_9. This should make it possible to estimate the

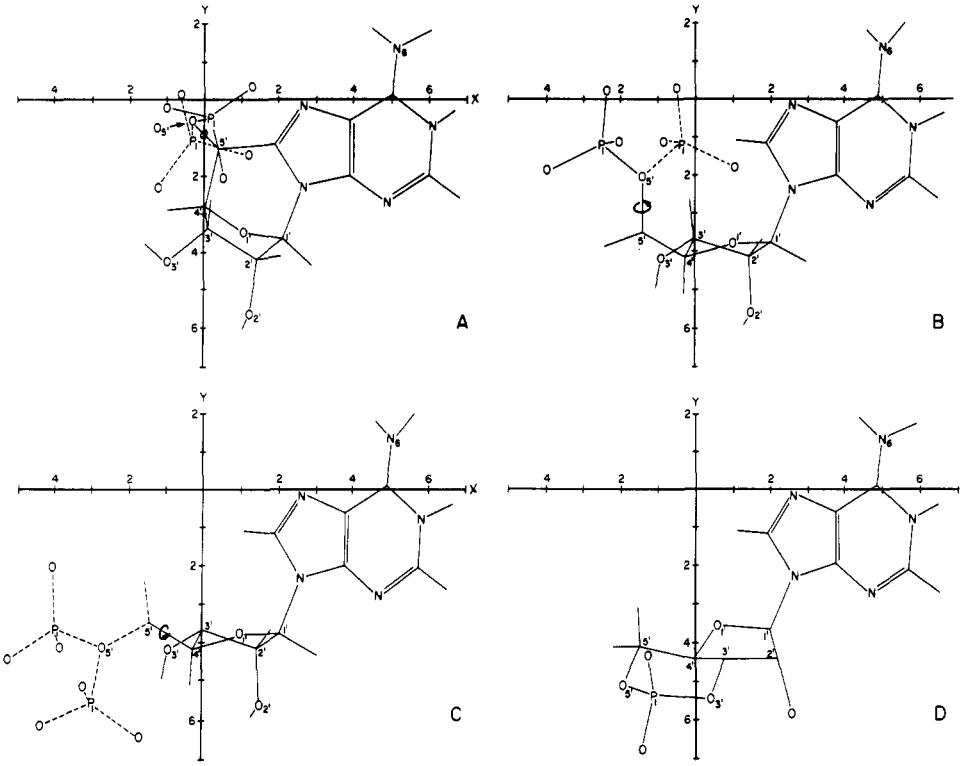


FIGURE 4: Interatomic distances for (A) (*S*)-8,5′-cycloAMP (Haromy et al., 1986), (B and C) 5′-AMP (Kraut & Jensen, 1963), and (D) 3′,5′-cAMP. Coordinates for 3′,5′-cyclic GMP (Chwang & Sundaralingam, 1973), transformed to the anti conformation, were used to approximate the structure of 3′,5′-cAMP. The three molecules, all in the anti conformation, were superimposed on an Evans and Sutherland modeling system by using a least-squares procedure, to minimize the distances between atoms N_1, N_7, and N_9 on the three respective adenine rings. Solid lines designate atomic positions based on crystal coordinates while dashed lines represent predicted atomic positions after rotation by 180° about the designated bond.

effect of interatomic distances between the 6-amino group and the 5′-phosphate (P_1) on binding of the compounds to the enzyme. Allowing for free rotation about the $C_{5'}$–$O_{5'}$ bond, P_1 is in about the same position in (*S*)-8,5′-cycloAMP (Figure 4A) and in 5′-AMP (Figure 4B), consistent with the ability of both molecules to bind tightly to the enzyme. The phosphoryl group of 3′,5′-cAMP (Figure 4D), an analogue that does not affect enzyme activity (Table II), is far removed from the coordinate location of P_1 of the active compounds shown in panels A and B of Figure 4. It should be noted, however, that random rotation about the $C_{4'}$–$C_{5'}$ bond in 5′-AMP would result in a position of P_1 (Figure 4C) that is closer to the coordinate location of P_1 in 3′,5′-cAMP (Figure 4D). If so, this would make it difficult to predict why 3′,5′-cAMP does not activate the enzyme. However, there is considerable evidence that the solution structure of 5′-AMP (Hampton et al., 1972) is quite similar to the crystal structure (Kraut & Jensen, 1963), and ^{1}H NMR (Schweizer et al., 1968; Danyluk & Hruska, 1968) and ^{31}P magnetic resonance studies (Tsuboi et al., 1968) suggest that, in solution, P_1 is positioned trans with respect to $C_{4'}$ as shown in Figure 4B. 3′,5′-cAMP and the 5′-phosphoramidate derivatives each contain one potentially ionizable hydroxyl on the phosphate. The positive effector activity of the phosphoramidate derivatives may be due to their ability to rotate about the $C_{4'}$–$C_{5'}$ bond to assume a coordinate position for P_1 that is similar to the configuration of 5′-AMP in panel B of Figure 4, whereas the 3′,5′-phosphodiester bond locks the cyclic compound into the unfavorable configuration of Figure 4D.

The secondary phosphate ester at carbon 5′ in (*S*)-8,5′-cycloAMP instead of the primary location of the phosphate in 5′-AMP may cause misalignment of (*S*)-8,5′-cycloAMP on binding to the enzyme, resulting in inhibition of activity. However, the symmetry of the opposites in effector activities obtained with (*S*)-8,5′-cycloAMP (inhibition) and 5′-AMP (activation) and the kinetic relationship between these compounds (Table II) suggest a more specific mechanism. For example, if one makes the usual assumption that an allosteric enzyme can exist in catalytically less competent (R) and more competent (T) forms, it is possible that the initial binding of (*S*)-8,5′-cycloAMP (or of 5′-AMP with a similar orientation of P_1 and the 6-amino group) occurs to the *R* form of the isocitrate dehydrogenase. Limited rotation about the $C_{4'}$–$C_{5'}$ bond may occur after 5′-AMP is bound to the R conformers of the enzyme, causing its conversion to the active T form. Since a rotation around the $C_{4'}$–$C_{5'}$ bond is prohibited in (*S*)-8,5′-cycloAMP, its binding would favor displacement of the enzyme to the R form, consistent with the observed action of (*S*)-8,5′-cycloAMP as a negative effector (Table II). A species of 5′-AMP retaining the configuration of (*S*)-8,5′-cycloAMP (Figure 4 A) and its tight binding properties for the enzyme would be advantageous for the sequential mechanism of enzyme activation proposed above, especially, if such a species of AMP occurred at relatively low concentration in solution. It may be relevant in this connection that, in studies of the relationship of structure to substrate function of 5′-AMP, the analogue (*R*)-8,5′-cycloAMP was a more effective substrate than noncyclized 5′-AMP for adenylate kinase (Hampton et al., 1972).

There are considerable similarities in structural requirements for the purine nucleotides which are allosteric modifiers for the NAD-specific isocitrate dehydrogenases from yeast (AMP) and bovine heart (ADP). Thus, for the mammalian enzyme (Plaut et al., 1979), an unsubstituted 6-amino group, an unsubstituted nitrogen 1, and the secondary phosphate of the 5′-pyrophosphoryl group of ADP were required for activation.

Effector activity is retained when an oxygen function is replaced by sulfur or nitrogen in the 5′-phosphoryl group of AMP for the yeast dehydrogenase and in the β-phosphate of the 5′-pyrophosphoryl group of ADP for the heart enzyme. As with the activation of the yeast enzyme by AMP, the nature of the substituents at carbons 2′ and 3′ of the pentosyl portion of ADP was not critical for the mammalian enzyme. The hydrogen at carbon 2 of AMP and ADP was not essential for activation of either the yeast or animal enzyme, respectively, and nitrogen 7 in either nucleotide could be replaced by carbon (i.e., the respective tubercidin analogues) with retention of enzyme activation. The formycin analogue of AMP was a weak positive effector of the yeast enzyme while this analogue of ADP was an inhibitor competitive with ADP for the dehydrogenase from heart.

ACKNOWLEDGMENTS

We gratefully acknowledge the gifts of Drs. N. J. Leonard, D. C. B. Mills, B. Haley, R. J. Suhadolnik, and J. A. Raleigh. We thank Dr. J. Glusker, Fox Chase Cancer Institute, for her help with molecular modeling and R. Colamesta for her help with the enzyme purification.

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