Dehydrogenase Binding by Tiazofurin Anabolites

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Thiazole-4-carboxamide adenine dinucleotide (TAD) is the active anabolite of the new antitumor agent tiazofurin (NSC 286193). TAD is an analogue of NAD in which the nicotinamide ring has been replaced by a thiazole-4-carboxamide heterocycle. TAD putatively acts by inhibition of inosine monophosphate dehydrogenase (IMPd). In this study it is shown that TAD is a competitive inhibitor, with respect to NAD, of mammalian glutamate, alcohol, lactate, and malate dehydrogenases. TAD binds to these enzymes with 1–2 orders of magnitude less affinity than it binds to IMPd. Computer modeling studies suggest that dehydrogenase binding by TAD occurs at the regular cofactor site, the thiazole-4-carboxamide group mimicking the steric and hydrogen-bonding properties of the nicotinamide ring. Noncompetitive kinetics of TAD inhibition of the target enzyme IMPd are potentially due to a reverse order of addition of substrate and cofactor from that observed in the dehydrogenases studied here. The weaker binding of TAD to these dehydrogenases may be due to their inability to preserve a close sulfur–oxygen contact in the bound inhibitor.

Tiazofurin (2-β-D-ribofuranosylthiazole-4-carboxamide, NSC 286193, Figure 1a) is a *C*-glycosyl thiazole nucleoside which has demonstrated significant antitumor activity in a number of model tumor systems. Tiazofurin is curative in vivo for the murine Lewis lung carcinoma.[1] It shows in vitro activity against human lymphoid, lung, and colon tumor cell lines, both alone and in combination with other oncolytic nucleoside analogues.[2-4] Although initial clinical trials of tiazofurin have demonstrated a variety of dose-limiting toxic side effects, recent findings suggest efficacy in the treatment of acute myeloid leukemia.[5]

The oncolytic activity of tiazofurin appears related to its ability to depress guanine nucleotide pools, with a subsequent interruption of DNA and RNA synthesis. This results from a decline in the guanosine precursor xanthosine monophosphate via inhibition of inosine monophosphate dehydrogenase (IMPd[31]).[6] The major IMPd inhibitor is a dinucleotide anabolite of tiazofurin. In vivo, tiazofurin is converted, via its 5′-phosphate (TrMP, Figure 1b), into an analogue of the cofactor nicotinamide adenine dinucleotide (NAD). In this NAD analogue, called TAD (thiazole-4-carboxamide adenine dinucleotide, Figure 1c), the nicotinamide ring is replaced by a thiazole-4-carboxamide moiety. TAD is a more potent inhibitor of IMPd than either TrMP or tiazofurin itself.[7]

One model for the mechanism of TAD inhibition of IMPd is that the dinucleotide analogue binds at the NAD(H) binding site, the heterocyclic base occupying the pocket normally filled by the nicotinamide ring.[8,9] However, no X-ray structure of IMPd has been obtained, thus the specific stereochemical requirements of the nicotinamide end of the cofactor binding site(s) on IMPd are unknown. Nevertheless, many features of NAD binding are common to a number of dehydrogenases of known structure.[10] Thus, TAD might be expected to inhibit other dehydrogenases in addition to IMPd. In order to test this hypothesis, enzyme inhibition and modeling studies have been initiated. Enzymes examined were bovine liver glutamate dehydrogenase (GDH), horse liver alcohol dehydrogenase (ADH), pig heart cytoplasmic malate dehydrogenase (MDH), and pig heart lactate dehydrogenase (LDH). Inhibition assays were carried out with TAD as inhibitor. Binding of the monophosphate precursor TrMP (Figure 1b) and the parent compound tiazofurin (Figure

Table I. Inhibition Constants (K_i, μM) for TAD and Its Precursors Using Various Dehydrogenases

	GDH	ADH	LDH	MDH
TAD	4.5 ± 0.6	26.5 ± 3.1[b]	25.2 ± 3.5	36.3 ± 3.2
TrMP	65.2 ± 6.1	105.0 ± 12.2	ND	ND
TF	>10⁴[a]	>10⁴[a]	ND	ND
NAD (K_m)	20 ± 0.82	22.3 ± 1.2	84.4 ± 7.6	33.9 ± 2.2
NMN	>10⁴[a]	>10⁴[a]	ND	ND

[a] Values for K_i for TF and NMN are lower limits estimated on the basis of the very slight slope effect shown in Figure 3 for TF and seen in similar studies for NMN. The slopes are not statistically different from those obtained in the absence of the inhibitor. ND = no data. [b] Measurement of direct binding of TAD to ADH via fluorescence quenching yields a dissociation constant (K_D) of 27 (6) μM.

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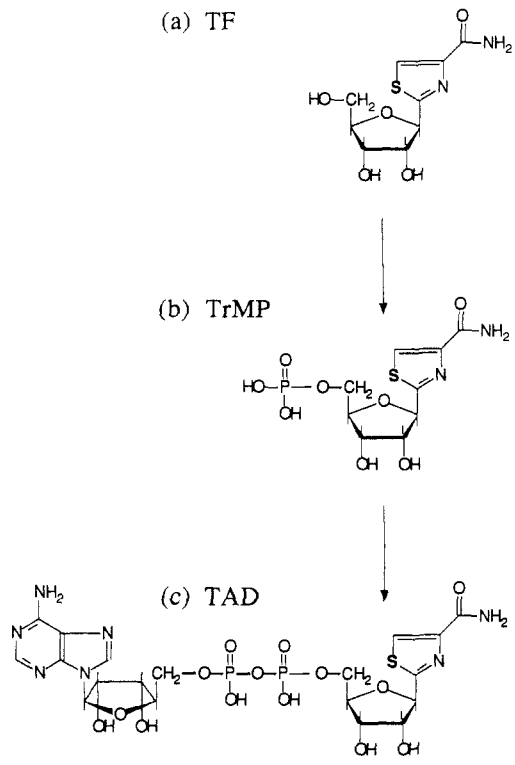

Figure 1. Tiazofurin (TF) and its anabolites TrMP and TAD.

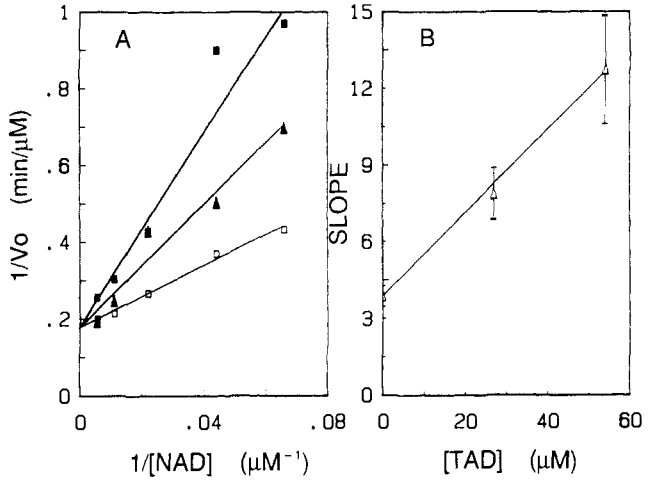

Figure 2. Inhibition of alcohol dehydrogenase using varied concentrations of TAD: (A) NAD concentrations varied at a fixed ethanol concentration of 1 mM. Assays were performed as described in the Methods section. Assays were performed at pH 8.0 using 5 μg/mL ADH in the absence of TAD (□) or in the presence of 27 μM TAD (▲) or 54 μM TAD (■). (B) Secondary plot of slopes from the primary plots in part A as a function of [TAD].

1a) were also examined. For comparison, measured K_m's of NAD and K_i's of nicotinamide mononucleotide (NMN)

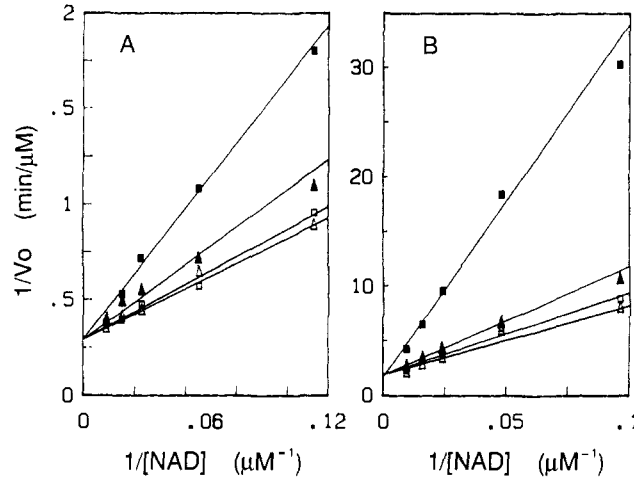

Figure 3. Inhibition of alcohol dehydrogenase and glutamate dehydrogenase by TAD and its precursors. (A) Alcohol dehydrogenase. NAD concentrations were varied in the presence of a fixed concentration of ethanol (1 mM) in the absence of inhibitors (△), or the presence of 15 μM TAD (■), 50 μM TrMP (▲), or 1 mM TF (□) with 2.5 μg/mL ADH. (B) Glutamate dehydrogenase. NAD concentrations were varied in the presence of 20 mM glutamate in the absence of inhibitors (△) or the presence of 15 μM TAD (■), 25 μM TrMP (▲), or 1 mM TF (□) with 3.3 μg/mL GDH.

were obtained as well. Experimental work was accompanied by modeling studies of enzyme-bound TAD. Results indicate that TAD does inhibit ADH, GDH, LDH, and MDH (albeit with less affinity than that reported for IMPd) and suggest that the dinucleotide analogue mimics NAD binding at the cofactor site.

Results and Discussion

Kinetic Studies. A Lineweaver–Burk plot for ADH with [NAD] varied in the presence of several concentrations of TAD is shown in Figure 2a. Slopes from this plot are shown as a function of [TAD] in Figure 2b. It is apparent that TAD acts as a competitive inhibitor of ADH with respect to NAD. Similar results are obtained for GDH, MDH, and LDH (data not shown). Failure of previous studies to show inhibition of these dehydrogenases by TAD was likely due to the use of crude tumor extracts as enzyme sources.[24] Such extracts would be expected to contain high levels of NAD glycohydrolase activity.[29] In GDH, TAD also showed noncompetitive

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inhibition with respect to glutamic acid (K_i = 5.7 μM). GDH follows a rapid equilibrium random order mechanism.[14] In this case, noncompetitive inhibition with respect to substrate further implies that TAD is a true cofactor analogue.[15] Kinetic constants are summarized in Table I. TAD shows competitive inhibition with respect to NAD in each dehydrogenase examined. In all cases, enzyme binding of the cofactor analogue is comparable to or tighter than that of NAD. These findings suggest that TAD mimics NAD binding at the cofactor site. This hypothesis is further supported by results shown in Figure 3.

Figure 3 compares inhibition by the dinucleotide analogue TAD, its monophosphate precursor TrMP, and the parent compound tiazofurin. Double-reciprocal plots for both ADH and GDH are shown with [NAD] as the variable substrate. Kinetic constants derived from these studies are also given in Table I. Like TAD, the mononucleotide analogue TrMP shows competitive inhibition with respect to NAD. TrMP binds less tightly than the dinucleotide TAD but more tightly than the parent compound tiazofurin. These are the results expected if one considers first the loss of the adenosine moiety (TAD → TrMP) and then the 5′-phosphate group (TrMP → tiazofurin) in stabilizing binding to the cofactor pocket side chains. Table I also shows K_i's for NMN from GDH and ADH. A comparison of these values shows that TrMP binding relative to NAD is at least 2–3 orders of magnitude tighter than that of nicotinamide mononucleotide (NMN). This suggests that the enzyme–monophosphate complex is further stabilized by the presence of the uncharged thiazole ring.

Modeling Studies. Kinetic data suggest that TAD mimics NAD binding at the cofactor site. In this case the thiazole-4-carboxamide moiety may occupy the pocket normally filled by the nicotinamide ring. This hypothesis is supported by modeling studies, which indicate that the steric and hydrogen bonding properties of the thiazole-4-carboxamide heterocycle closely approximate those of the nicotinamide ring.

Among the dehydrogenases examined, the structure of GDH is unknown, although the kinetic data do suggest that TAD is binding at the high-affinity catalytic site.[21] However, the structure of horse liver alcohol dehydrogenase has been extensively studied and the cofactor binding site is well-characterized.[16] Only one NAD binding site exists per subunit, hence TAD presumably binds to this site. On the basis of this assumption, a model of ADH-bound TAD is proposed.

The cofactor binding site on horse liver ADH is characterized by specific interactions at the nicotinamide end. The nicotinamide moiety is anchored in an interior pocket of the enzyme by three hydrogen bonds to its carboxamide group. The carboxamide oxygen acts as a hydrogen-bond acceptor from the main chain nitrogen of residue Phe-319. The carboxamide amino group donates H bonds to carbonyl oxygens on Ala-317 and Val-292.[16] These interactions are conserved in four ADH complexes with NAD and various coligands[16] and thus might be expected to be conserved in TAD binding to ADH.

Figure 4 shows a model of ADH-bound TAD. This was obtained by performing a least-squares fit between the thiazole-4-carboxamide moiety and the nicotinamide ring, followed by replacement of this ring with the thiazole heterocycle in the ternary complex. Coordinates were obtained, via the Protein Data Bank, from the ternary complex of horse liver ADH with cofactor and dimethyl

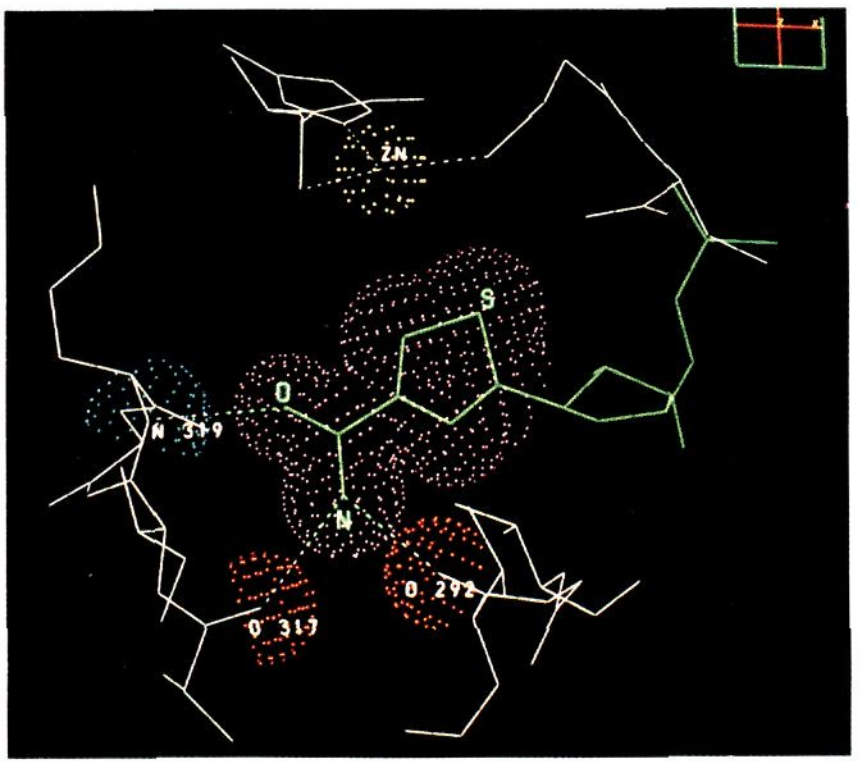

Figure 4. Model of TAD binding to ADH. The TAD backbone is shown in green. The protein backbone is white. Van der Waals surfaces are drawn at $^4/_5$ van der Waals radii and are colored as follows: thiazole-4-carboxamide moiety, magenta; catalytic Zn, yellow; backbone nitrogen of residue 319, blue; carbonyl oxygens of residues 317 and 292, orange. The carboxamide oxygen acts as a potential hydrogen bond acceptor from N319. The carboxamide amino group acts as a potential H-bond donor to O317 and O292.

Figure 5. Overlap of nicotinamide and thiazole-4-carboxamide groups, each surrounded by its van der Waals surface. The thiazole ring (orange) occupies 92% of the volume of the nicotinamide ring (white).

sulfoxide.[16] The three potential hydrogen bonds to the carboxamide group on TAD closely mimic those formed by the nicotinamide moiety on NAD. In forming these bonds, the carboxamide NH_2 group remains cis to the thiazole ring nitrogen. Crystal structures of a number of tiazofurin analogues indicate that this is the low-energy conformation of the carboxamide substituent.[9] The thiazole ring itself fits well into the nicotinamide binding pocket, forming no unfavorable contacts with neighboring side chains. However, in order to obtain the fit shown in Figure 4, the thiazole ring must be rotated about the C–glycosyl bond by ~50° relative to the conformation observed in the crystal structure of the parent compound. Potential implications of this stereochemical requirement are discussed below. In this model, the catalytic zinc–thiazole sulfur contact (~3.9 Å) roughly preserves the

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geometry characteristic of an electrophile–sulfur interaction.[22]

In a similar fashion, models may be constructed of LDH- and MDH-bound TAD employing crystal structures of these dehydrogenases.[18,19] These models (not illustrated) share the same features as those described above. In both cases the thiazole ring is easily accommodated by the nicotinamide binding pocket. The thiazole's carboxamide group may be positioned to reproduce all nicotinamide side chain interactions while remaining in its low-energy conformation (NH_2 cis to thiazole N). Thus, the thiazole-4-carboxamide moiety may mimic nicotinamide binding in each of the dehydrogenases examined. This is a consequence of the similar sizes of the two rings and the similar relative location of their carboxamide substituents. This is illustrated in Figure 5, in which the steric properties of the nicotinamide and thiazole-4-carboxamide rings are compared. The thiazole ring occupies over 90% of the volume of the nicotinamide ring. However, despite the steric similarities, the chemistry of the two heterocycles is different. The thiazole ring is highly resistant to reduction.[23] Thus, TAD is unlikely to act as a hydride ion acceptor, even if bound in a ternary complex.

Implications for IMPd Binding. As described above, the carboxamide group on the thiazole ring may allow the cofactor analogue TAD to mimic NAD binding at the nicotinamide end of the cofactor pocket. Tiazofurin requires the carboxamide group in the 4-position of the thiazole ring for activity.[6] This suggests that the tiazofurin anabolite TAD may bind to the target enzyme IMPd at the cofactor site as well. TAD is a noncompetitive inhibitor of IMPd with respect to NAD.[24-26] However, it should be emphasized that noncompetitive inhibition of IMPd by TAD with respect to NAD will be observed if TAD mimics NAD binding at the regular cofactor site.

IMPd from a variety of sources follows an ordered sequential mechanism in which substrate binds first, followed by cofactor.[26] If TAD bound only in the absence of substrate, uncompetitive inhibition with respect to NAD would be observed.[27] If TAD bound only in the presence of substrate, purely competitive inhibition with respect to NAD would be observed.[27] Observation of noncompetitive kinetics with respect to NAD implies only that both enzyme–TAD and enzyme–TAD–substrate complexes are formed (Figure 6a). Thus, noncompetitive kinetics would be observed if TAD bound IMPd at the cofactor site both in the presence and absence of substrate.

Observation of competitive kinetics in the dehydrogenases studied here does not necessarily imply a different mode of interaction with TAD. ADH, LDH, and MDH all follow ordered sequential mechanisms in which cofactor binds first, followed by substrate[28] (Figure 6b). In these enzymes, TAD may also bind both in the presence and absence of substrate. However, a sufficiently large increase in [NAD] will block formation of both enzyme–TAD and enzyme–TAD–substrate complexes. Thus, these enzymes show purely competitive inhibition by TAD with respect to NAD. A similar effect is seen in GDH as a result of its rapid equilibrium random order mechanism.[14,27] Hence, in both the dehydrogenases studied here and the target enzyme IMPd, the basic mechanism of inhibition is likely the same: the thiazole-4-carboxamide group on TAD mimics cofactor binding at the nicotinamide end of the binding pocket.

Details of TAD binding at the cofactor site on IMPd are clearly different from those modeled for ADH, LDH, and MDH. This is indicated by a difference in relative K_i's. The most recent kinetic study of IMPd inhibition by TAD, using highly purified enzyme from rat hepatoma 3924A,

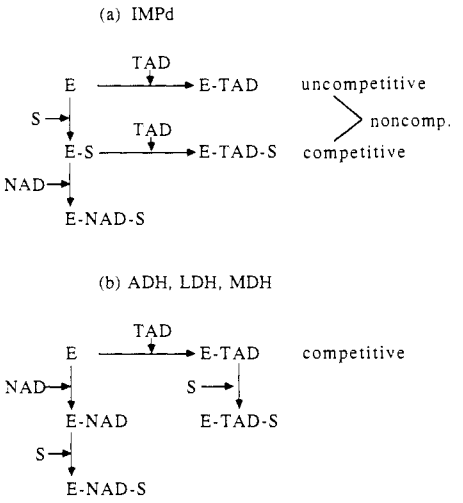

Figure 6. Proposed mechanisms of TAD inhibition of IMPd (a) and ADH, MDH, and LDH (b). E = IMPd (a) or E = ADH, MDH, LDH (b). S = substrate.

showed that TAD is a noncompetitive inhibitor of mixed type with respect to NAD.[26] Kinetic constants were K_i (slope) = 0.73 μM and K_{ii} (intercept) = 0.13 μM,[26] 1–2 orders of magnitude less than the values obtained for TAD binding to GDH, ADH, LDH, and MDH (Table I). The K_m for IMPd-bound NAD is 65 μM,[26] comparable to the K_m's for NAD shown in Table I. Thus, TAD binds with greater affinity to IMPd than to the enzymes studied here. This may in part be due to the specific stereochemical requirements of the cofactor binding site on each enzyme, as discussed next.

Crystal structures of tiazofurin and its analogues show conservation of close contacts between the thiazole sulfur and the furanose ring oxygen. These have been attributed to an attractive electrostatic S···O1′ interaction.[8,9,30] This interaction is compromised in the models of ADH-, LDH-, and MDH-bound TAD discussed above. The model of ADH-bound TAD requires a 50° rotation in the glycosidic torsion angle relative to that found in the crystal structure of tiazofurin. Models of TAD bound to MDH and LDH require greater rotations about the glycosidic bond, further increasing the sulfur–oxygen distance. Preliminary computational studies[30] indicate that these conformational changes would result in an increase in binding energy of 2–3 kcal/mol. This is sufficient to account for the observed 1–2 order of magnitude increase in K_i's, even in the absence of entropic effects. Thus, the lower affinity of ADH, LDH, and MDH for TAD may be due to the inability of these enzymes to sterically accommodate the thiazole ring *and* maintain the sulfur–oxygen interaction. Conversely, the conformation of IMPd-bound TAD may preserve the S···O1′ interaction, accounting for the significantly lower K_i seen with this target enzyme.

Summary and Conclusions

Kinetic studies show that TAD, the active anabolite of the antitumor drug tiazofurin, is a competitive inhibitor of GDH, ADH, LDH, and MDH with respect to NAD.

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(31) The abbreviations used are as follows: TF, tiazofurin; TrMP, thiazole-4-carboxamide 5′-monophosphate; TAD, thiazole-4-carboxamide adenine dinucleotide; IMPd, inosine monophosphate dehydrogenase; GDH, bovine liver glutamate dehydrogenase; ADH, equine liver alcohol dehydrogenase; MDH, porcine heart cytoplasmic malate dehydrogenase; LDH, porcine heart lactate dehydrogenase.

Modeling studies suggest that TAD inhibits these dehydrogenases by binding at the cofactor site, the thiazole-4-carboxamide group mimicking the steric and hydrogen-bonding properties of the nicotinamide ring. It is plausible that TAD binds to the putative target enzyme IMPd in a similar fashion, given that many features of cofactor binding are conserved among the dehydrogenases. Binding of TAD to IMPd differs from that observed for GDH, ADH, LDH, and MDH in two ways. Kinetics of inhibition of IMPd by TAD with respect to NAD are noncompetitive, and binding of TAD to IMPd is tighter. Differences in kinetics may result from the difference in order of addition of substrate and cofactor to IMPd. The tighter binding of TAD to IMPd may in part be due to this enzyme's ability to maintain an attractive sulfur–oxygen interaction in the bound conformation of the inhibitor. In either case, it seems likely that TAD binds to IMPd by mimicking cofactor binding. Thus, in the design of new tiazofurin and/or TAD analogues, preservation of the size and hydrogen-bonding abilities of the nicotinamide end of the native coenzyme may be a necessary (although not sufficient) condition for activity.

Experimental Section

Materials. TAD was synthesized as the free acid by the methods described by Gebeyehu et al.[11] Tiazofurin and TrMP were prepared according to the methods of Srivastava et al.[12] Horse liver alcohol dehydrogenase was obtained from Sigma Chemical Co., St. Louis, MO, in the lyophilized crystalline form. Pig heart lactate dehydrogenase and the cytoplasmic form of malate dehydrogenase were also from Sigma and were obtained as suspensions in ammonium sulfate. Bovine glutamate dehydrogenase was obtained as a solution in 50% glycerol. All enzymes were diluted to the required stock concentrations with a stock buffer of the same pH and composition as the stock buffer used for each series of assays. Concentrations of the stock enzyme solutions were determined spectrophotometrically with published extinction coefficients at 280 nm.[13,28] Enzyme solutions were made on the day of use and kept on ice during the course of the kinetic experiments.

Cofactors (NAD, NADH, and NMN) were obtained from Sigma Chemical Co. Concentrations of stock solutions were determined spectrophotometrically with published extinction coefficients. Substrates were obtained from Sigma and prepared fresh for use. Concentrations of substrate solutions were calculated from weight and the molecular weight of the compound. All solutions were made up with 18 MΩ water from a Millipore system.

Kinetic Studies. Rate measurements for each of the dehydrogenases used in this study are based on the spectral properties of NADH. In assays with NAD as a substrate, rates were determined by measuring the increase in absorbance at 340 nm or by following the appearance of fluorescence (using an excitation wavelength of 340 nm and monitoring emission at 450 nm) resulting from the conversion of NAD to NADH. Rates using absorbance measurements were calculated with a millimolar extinction coefficient of 6.22/cm for NADH. Rates from the fluorescence experiments were calibrated with standard NADH concentrations measured under the same conditions.[14]

Alcohol dehydrogenase assays were run at pH 8.0, using 0.1 M sodium phosphate buffer. Glutamate dehydrogenase assays were at pH 7.0 in 0.1 M sodium phosphate buffer, containing 10 μm EDTA. Malate dehydrogenase and lactate dehydrogenase assays were performed at pH 9.0 with 0.05 M Tris HCL buffer. All kinetic assays were run at least in duplicate.

Lineweaver–Burk plots were used to analyze the kinetic data, using linear regression to obtain values for the slope and intercept of each line. Inhibition was judged to be competitive if the values obtained for the intercepts of the appropriate plots differed by less than 1 standard deviation as determined by linear regression. Values for the inhibition constant, K_i, were obtained with the relationship[15]

$$\text{slope}(+I) = \text{slope}(-I)(1 + [I]/K_i)$$

K_m for coenzyme was obtained from the slope and intercept obtained from plots in the absence of inhibitor, with the relationship

$$K_m = \text{slope}/V_{max}$$

Modeling Studies. The starting point for the model of ADH-bound TAD was the crystallographic study of the ternary complex of equine liver ADH with cofactor and dimethyl sulfoxide.[16] Coordinates were obtained from the Brookhaven Protein Data Bank.[17] A least-squares fit was performed between the nicotinamide moiety in the ternary complex and the thiazole-4-carboxamide group from the crystal structure of tiazofurin.[8] Rotations about the carboxamide bond were permitted. This allowed the carboxamide substituent on the thiazole ring to best approximate the hydrogen bonds formed by the nicotinamide moiety in the original complex. Only distances between atom pairs were minimized, without explicit weighting by energy terms. The nicotinamide moiety in the original structure was then replaced by the thiazole-4-carboxamide group. A model of LDH-bound TAD was derived in a similar fashion from the 2.7-Å structure of pig heart lactate dehydrogenase complexed with the coenzyme–substrate analogue S-lac-NAD.[18] Coordinates were obtained from the Protein Data Bank. A model of MDH-bound TAD was also constructed, utilizing recently refined 2.5-Å data from a binary complex of cytoplasmic pig heart malate dehydrogenase with cofactor.[19] Coordinates were kindly supplied by Drs. J. Birktoft and L. Banaszak. The CHEMX system of programs[20] was employed in each case. Software was installed on a Microvax II computer driving an Evans and Sutherland PS300 graphics device.

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