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USE OF A BISUBSTRATE INHIBITOR TO DISTINGUISH BETWEEN ISOCITRATE DEHYDROGENASE ISOZYMES

ROBERT S. EHRLICH*

Department of Chemistry and Biochemistry, University of Delaware, Newark, DE 19716, USA

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Bisubstrate inhibitors, obtained by covalently linking 2-oxoglutarate with NAD^+ and $NADP^+$, were synthesized and tested for their ability to inhibit NAD^+- and $NADP^+$-dependent isocitrate dehydrogenases from pig heart mitochondria. The $NADP^+$-dependent enzyme was specifically inhibited by the NADP–oxoglutarate adduct and not by the NAD adduct. The NADP adduct was competitive with both coenzyme and substrate, isocitrate. In contrast, the NAD^+-dependent enzyme was inhibited by both adducts. NAD–oxoglutarate is competitive with both NAD^+ and isocitrate while the NADP adduct is competitive with isocitrate but not with NAD^+. Nevertheless conditions could be set up so that use of these inhibitors would be feasible for a metabolic study.

Keywords: Isocitrate dehydrogenase; Oxoglutarate; NAD(P)H; Inhibitor; Coenzyme

INTRODUCTION

Study of the metabolic roles of enzymes may be facilitated by the use of specific inhibitors. Double headed inhibitors that combine features of two substrates of an enzyme could enhance this specificity. Such inhibitors are exemplified by the covalently linked coenzyme-adducts originally synthesized by Everse *et al.*[1] Combination of NAD^+ with different substrates has been proposed as a technique for distinguishing among the activities of glutamate dehydrogenase by Marchand *et al.*[2] but the specificity has been challenged by Syed and Engel.[3] In the current study an attempt has been made

* Tel.: 302-478-8135. Fax: 302-831-6335. E-mail: robehrlich@aol.com.

to see if coenzyme–substrate adducts can selectively inhibit isocitrate dehydrogenase isozymes.

Pig heart mitochondria contain two isozymes of isocitrate dehydrogenase distinguished by specificity[4] for the coenzyme NAD^+ or $NADP^+$. Both enzymes require divalent metal for the oxidative decarboxylation of isocitrate to yield 2-oxoglutarate. Adducts of NAD^+ and $NADP^+$ with 2-oxoglutarate were synthesized and each was tested for inhibitory properties with respect to both the enzyme forms utilizing the appropriate coenzyme and the enzyme forms using the other substrate.

MATERIALS AND METHODS

$NADP^+$-dependent isocitrate dehydrogenase was purified from pig hearts as described by Ehrlich and Colman.[5] NAD^+-dependent isocitrate dehydrogenase was purified following the method described by Ehrlich and Colman.[6] The enzymes were dialyzed against the stabilizing buffers containing 10% glycerol for the $NADP^+$-dependent enzyme and 20% glycerol for the NAD^+-dependent enzyme. Enzymes were stored at −80°C and quickly thawed prior to assay. Coenzymes, 2-oxoglutarate and DL-isocitrate were obtained from Sigma.

The assay of NAD^+-dependent isocitrate dehydrogenase was performed in tris-33 mM acetate (pH 7.2) containing 1 mM manganese sulfate and concentrations of NAD^+ and DL-isocitrate as indicated under experimental results. $NADP^+$-dependent isocitrate dehydrogenase was assayed in 30 mM triethanolamine chloride (pH 7.4) with 2 mM manganese sulfate.

Covalent adducts of 2-oxoglutarate with NAD^+ and $NADP^+$ were prepared using the methods of Everse *et al.*[1] The adducts were separated from starting materials using chromatography on DE-52 (Whatman) with an ammonium bicarbonate gradient from 0.01 to 0.5 M. The products were characterized by proton NMR and UV-spectroscopy. The structure of the NAD adduct is shown in Figure 1. The concentrations were determined using a molar extinction of 8100 M^{-1} cm at 260 nm.[7] Compounds were lyophilized and stored at −20°C.

RESULTS

Inhibition of NAD^+-Dependent Isocitrate Dehydrogenase by NAD–Oxoglutarate

Figure 2A and B are double reciprocal plots of the activity of NAD^+-dependent isocitrate dehydrogenase with varying NAD^+ or isocitrate,

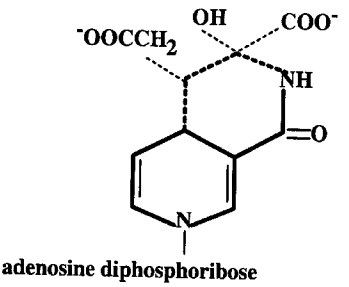

FIGURE 1 Structure of the NAD–oxoglutarate adduct. The solid lines represent bonds in the nicotinamide portion and the dashed lines bonds in the oxoglutarate moiety.

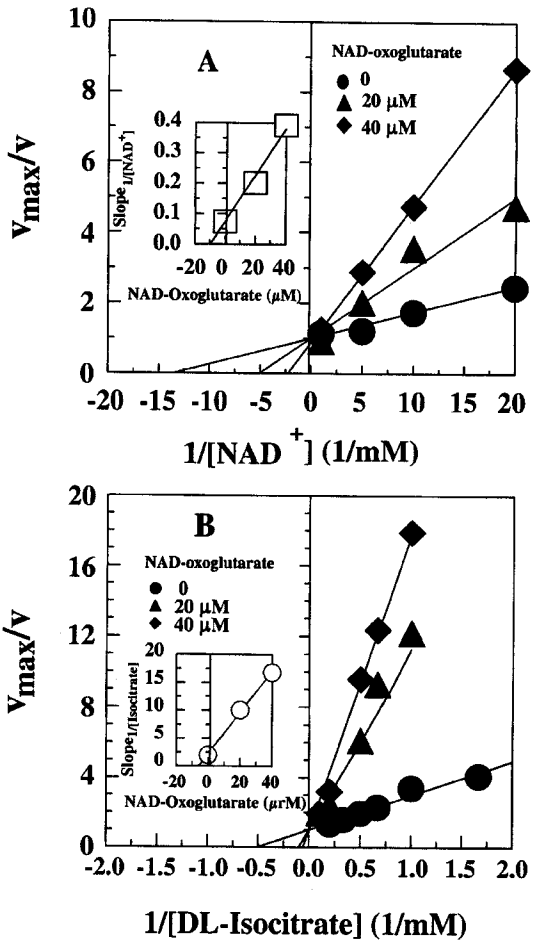

FIGURE 2 Double reciprocal plots of relative NAD-dependent isocitrate dehydrogenase activity in the presence of the indicated concentrations of NAD–oxoglutarate with varying, (A) NAD^+ and (B) isocitrate. The second substrate is 1 mM isocitrate or 100 μM NAD^+, respectively. Insets are plots of the slopes of the lines.

respectively. The intersection of lines, obtained by fitting data at different inhibitor concentrations, at the y-axis indicates that NAD–oxoglutarate is a competitive inhibitor with respect to NAD^+ and DL-isocitrate. Replots of the slopes of the lines (insets) fall on straight lines, indicative of linear competitive inhibition. The dissociation constant of NAD–oxoglutarate from NAD^+-dependent isocitrate dehydrogenase is calculated from the apparent dissociation constant defined by the intercept of the plots using:

$$K_{app} = K_i(1 + [S]/K_s) \quad (1)$$

where [S] is the concentration of the fixed substrate, NAD^+ or DL-isocitrate. From Equation (1) the calculated dissociation constant for NAD–oxoglutarate is $3.7 \pm 0.7\,\mu M$. This dissociation constant is an order of magnitude lower than the dissociation constant for NAD^+, $55\,\mu M$,[8] and comparable to the dissociation constant for NADH, $2\,\mu M$.[9,10] The observation that NAD–oxoglutarate is a competitive inhibitor with respect to both NAD^+ and isocitrate suggests that the adduct binds in a configuration that overlaps both sites. The enhanced binding of the bisubstrate adduct suggests interaction with both the substrate and coenzyme moieties of the active site but enhancement could be merely due to neutralization of the positive charge on the nicotinamide ring.

Inhibition of $NADP^+$-Dependent Isocitrate Dehydrogenase by NADP–Oxoglutarate

Figure 3A and B are double reciprocal plots of velocity with respect to the substrates for $NADP^+$-dependent isocitrate dehydrogenase. The data are consistent with this adduct acting as a competitive inhibitor with respect to both $NADP^+$ and isocitrate. Replots of the slopes (not shown) indicate linear competition with respect to both substrates. While the apparent K_i for NADP–oxoglutarate is $22\,\mu M$ from competitive inhibition against $NADP^+$ and $3.8\,\mu M$ from competitive inhibition with isocitrate, from Equation (1) an intrinsic binding constant of $0.30 \pm 0.04\,\mu M$ is calculated. As in the case of the NAD^+-dependent enzyme the enhanced binding could arise from additional adduct–enzyme interactions compared with the interactions of either substrate or coenzyme alone or it could arise from neutralization of the charge on the nicotinamide ring. Proton NMR studies have demonstrated that the configuration of the nicotinamide ring in the enzyme-bound complex is different for oxidized and reduced coenzymes.[11]

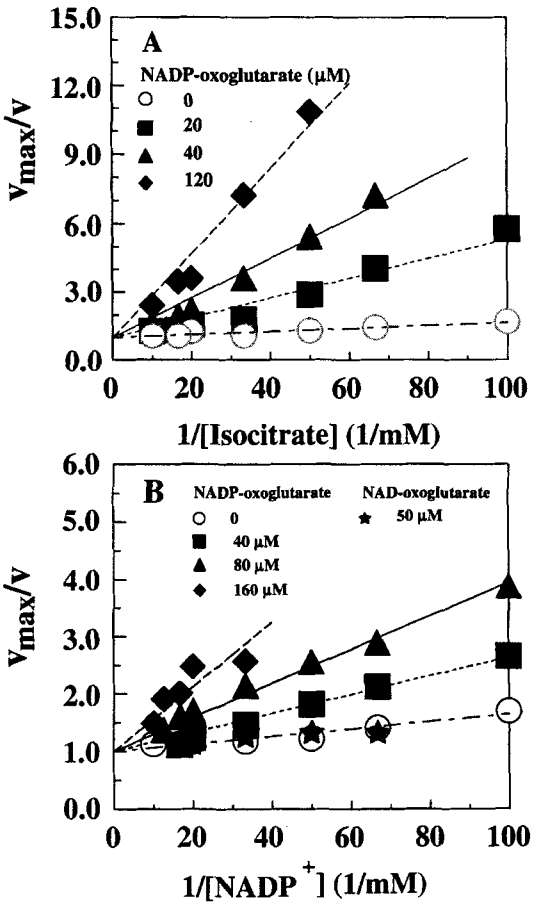

FIGURE 3 Double reciprocal plots of relative NADP-dependent isocitrate dehydrogenase activity in the presence of the indicated concentrations of NADP–oxoglutarate with varying (A) isocitrate and (B) $NADP^+$. The second substrate is 100 μM $NADP^+$ or 0.4 mM isocitrate. The activity when 50 μM NAD–oxoglutarate is present is also shown in (B) (filled stars).

Inhibition of $NADP^+$-Dependent Isocitrate Dehydrogenase by NAD–Oxoglutarate

Figure 3B also shows velocities measured when 50 μM NAD–oxoglutarate is present. The velocities measured are not statistically different than those measured in the absence of the adduct. The intrinsic dissociation constant of this adduct from the enzyme is greater than 25 μM. Thus the enzyme shows at least 100-fold discrimination between the $NADP^+$ and NAD^+ adducts. This is consistent with the strong discrimination between $NADP^+$ and NAD^+ as substrates.[12]

Inhibition of NAD$^+$-Dependent Isocitrate Dehydrogenase by NADP–Oxoglutarate

Figure 4A shows a double reciprocal plot of the velocity of NAD$^+$-dependent isocitrate dehydrogenase with respect to isocitrate. The inhibition appears competitive with an apparent K_i of 34 μM. When NAD$^+$ is the varied substrate the inhibition is not competitive (Figure 4B). The inhibition pattern is typical of a case where the inhibitor, NADP–oxoglutarate, competes with isocitrate while the substrate, NAD$^+$ binds with altered affinity

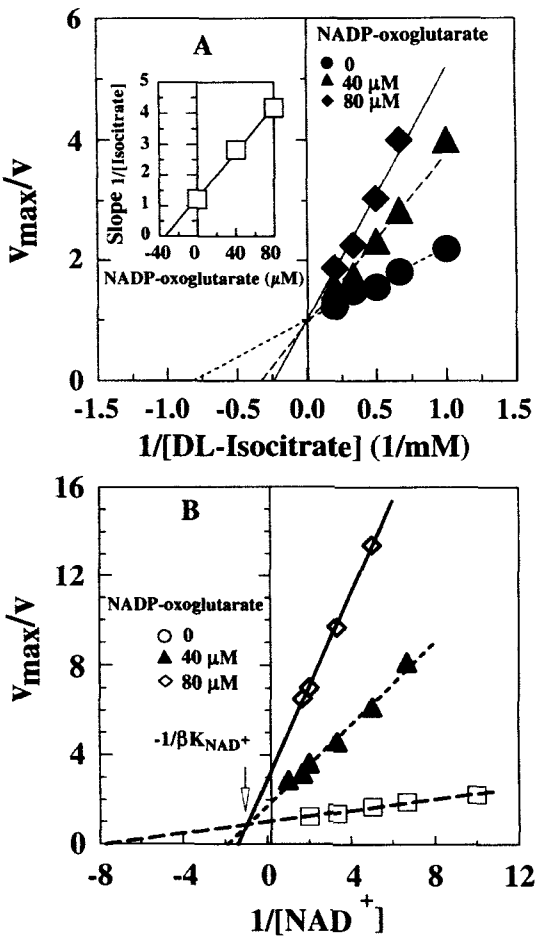

FIGURE 4 Double reciprocal plots of relative NAD-dependent isocitrate dehydrogenase activity in the presence of the indicated concentrations of NADP–oxoglutarate with varying (A) isocitrate and (B) NAD$^+$. The second substrate is 0.5 mM NAD$^+$ or 1 mM isocitrate.

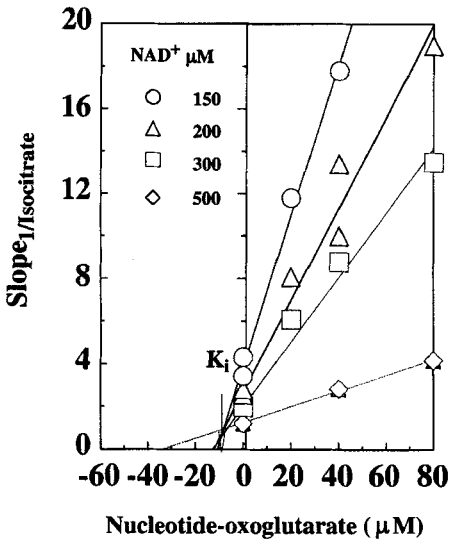

FIGURE 5 Replot of slopes from reciprocal plots of relative velocity versus isocitrate. Data from Figure 4A and similar data at other NAD^+ concentrations are included.

(by a factor β) to the enzyme:[13]

$$\text{E-inhibitor} + \text{NAD}^+ \overset{\beta K_{\text{NAD}^+}}{\longleftrightarrow} \text{E-inhibitor}-\text{NAD}^+ \qquad (2)$$

The affinity of the enzyme for NAD^+ is reduced almost 10-fold by the inhibitor ($\beta = 0.13$). The inhibition constant is obtained from a replot of the slopes of the lines in Figure 4A. Using this data and other data at different NAD^+ concentrations (Figure 5) the inhibition constant is $8.6 \pm 1.3\,\mu M$. This inhibition constant is comparable to the binding constant of NADPH to NAD^+-dependent isocitrate dehydrogenase ($8.1\,\mu M$).[10] NADPH is not competitive with NAD^+ or NADH but facilitates the inhibition by NADH.[9] The results obtained with the NAD–oxoglutarate adduct indicate that the adduct may bind in the region of isocitrate and alter the environment at the nucleotide site but that it does not directly occupy that site. A binding distal from the active site that alters the conformation at both isocitrate and NAD^+ moieties of the active site cannot be ruled out.

DISCUSSION

With $NADP^+$-dependent isocitrate dehydrogenase the strategy of using coenzyme–substrate adducts results in a strong selection for the

NADP-containing inhibitor as predicted by the substrate specificity of the enzyme. This inhibitor is competitive with both substrates and binds more tightly, as indicated by the inhibition constants, than either one alone. In contrast the NAD^+–oxoglutarate inhibitor does not show any inhibition at 50 μM.

With NAD^+-dependent isocitrate dehydrogenase, the NAD containing adduct is an effective inhibitor that may block both coenzyme and substrate sites. The NADP containing adduct also binds to the enzyme although the competition pattern indicates that it may bind only to the substrate site. The intrinsic binding constant for this inhibitor (8.6 μM) is only slightly higher than that of the NAD adduct (3.7 μM).

While the kinetic analysis of the performance of the two inhibitors with the two enzymes indicates that they would not yield complete discrimination between the isozymes, partial discrimination could be obtained by using a low concentration of the NADP–oxoglutarate adduct (i.e. between 0.3 and 8.6 μM) and, separately, a concentration of the NAD–oxoglutarate adduct of about 25 μM. Thus, the adduct would be useful in studying the enzymes in a metabolic system where both NAD^+ and $NADP^+$ are present.

Acknowledgements

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References

[1] J. Everse, E.C. Zoll, L. Kahan and N.O. Kaplan (1971) *Bioorg. Chem.*, **1**, 207–233.
[2] J. Marchand, J. Torreilles, M.-C. Guerin, B. Descomps, A.C. de Faulet, M. Gabriel and D. Larcher (1982) *Biochimie*, **64**, 203–209.
[3] S.E.-H. Syed and P.C. Engel (1993) *Biochem. Mol. Biol. Int.*, **30**, 283–291.
[4] R.S. Ehrlich and R.F. Colman (1992) *Biochemistry*, **31**, 12 524–12 531.
[5] R.S. Ehrlich and R.F. Colman (1983) *J. Biol. Chem.*, **258**, 7079–7086.
[6] R.S. Ehrlich and R.F. Colman (1990) *Biochemistry*, **29**, 5179–5187.
[7] J. Marchand, J. Torreilles, M.-C. Guerin, B. Descomps, A.C. de Faulet, M. Gabriel and D. Larcher (1982) *Biochim. Biophys. Acta*, **707**, 7–13.
[8] R.S. Ehrlich and R.F. Colman (1981) *J. Biol. Chem.*, **256**, 1276–1282.
[9] R.A. Harvey, J.I. Heron and G.W.E. Plaut (1972) *J. Biol. Chem.*, **247**, 1801–1808.
[10] R.S. Ehrlich and R.F. Colman (1982) *J. Biol. Chem.*, **257**, 4769–4774.
[11] R.S. Ehrlich and R.F. Colman (1985) *Biochemistry*, **24**, 5378–5387.
[12] R.S. Ehrlich and R.F. Colman (1978) *Eur. J. Biochem.*, **89**, 575–587.
[13] I.H. Segel (1975) *Enzyme Kinetics*, pp. 283–309. John Wiley; New York.