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UMP-DEPENDENT REDUCTION OF UDP-GALACTOSE 4-EPIMERASE–NAD^+ COMPLEX BY SODIUM CYANOBOROHYDRIDE

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SUMMARY

The reported spontaneous reoxidation of the UDPgalactose 4-epimerase (EC 5.1.3.2)–NADH complex prepared by $NaBH_4$ reduction of the native enzyme is oxygen dependent. Therefore, the ultimate electron acceptor in autoxidation is molecular oxygen rather than any functional or prosthetic group endogenous to the enzyme.

The epimerase–NAD^+ complex can be reduced by $NaBH_3CN$ in a reaction that is absolutely dependent upon the presence of UMP under either aerobic or anaerobic conditions. Therefore, the function of UMP in promoting this reaction is to increase the rate at which NAD^+ is reduced, rather than to stabilize the epimerase–NADH complex toward autoxidation.

The rate at which $NaBH_3CN$ reacts with the UMP-activated epimerase–NAD^+ complex is substantially faster under most conditions than the rate at which it reacts with NAD^+ itself under identical conditions.

INTRODUCTION

The tightly bound NAD^+ associated with UDPgalactose 4-epimerase from *Escherichia coli* or *Saccharomyces fragilis* is reducible by $NaBH_4$, but the inactive epimerase–NADH complex quickly undergoes a spontaneous reactivation reaction which is associated with the disappearance of NAD [1, 2]. It is also known that substrates or UMP stabilize the epimerase–NADH complex against reversion to an active form and that the *E. coli* epimerase is subject to UMP-dependent and substrate-dependent reductive inactivation by $NaBH_4$ [2–4].

RESULTS AND DISCUSSION

Spontaneous oxidation of the inactive epimerase–NADH complex to active epimerase–NAD^+ has been repeatedly reported to be a property of this enzyme, first by Creveling et al. [1] and later by others [2]. The established facts are that epimerase–NAD^+ can be reduced by $NaBH_4$ to inactive epimerase–NADH which then undergoes spontaneous reoxidation and reactivation [2]. In these experiments the only

possible electron acceptors for NADH are the enzyme itself, for example a disulfide linkage whose integrity is not essential for enzyme activity, or molecular oxygen. Fig. 1 establishes that oxygen is the ultimate electron acceptor. Upon reducing the *E. coli* epimerase–NAD^+ to epimerase–NADH with $NaBH_4$ and permitting it to reoxidize, the cycle of reduction and reoxidation can be repeated, which suggests that the protein is not itself the ultimate electron acceptor. Part B of Fig. 1 shows that epimerase–NADH is not spontaneously reoxidized under anaerobic conditions, but upon admission of air autoxidation proceeds. We conclude that the ultimate electron acceptor for epimerase–NADH is molecular oxygen.

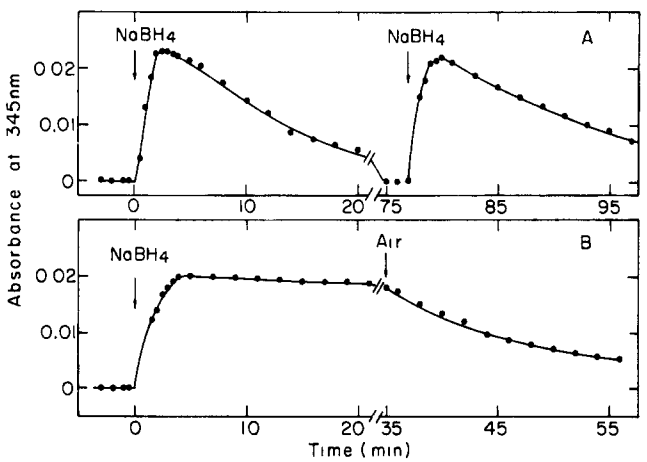

Fig. 1. Oxidation of epimerase–NADH by O_2. *E. coli* UDPgalactose 4-epimerase was purified by the procedure of Wilson and Hogness [5]. In Part A 5.3 μM enzyme (based on NAD^+ content) in 0.90 ml of 0.2 M sodium bicinate buffer at pH 8.50 in a semimicro cuvet were treated aerobically with 0.05 ml of 0.1 M $NaBH_4$ at the indicated times. The appearance and disappearance of NADH was monitored at 345 nm with a Norelco-Unicam SP 800 spectrophotometer. In Part B the complete reaction mixture consisted of 5.0 μM UDPgalactose 4-epimerase, 0.2 M sodium bicinate buffer, and 0.005 M $NaBH_4$ inside an anaerobic cuvet. With the enzyme and buffer in the cuvet and the $NaBH_4$ in the side arm, the solutions were flushed with N_2 gas. The reaction was started by tipping the $NaBH_4$ into the cuvet and the appearance of NADH was monitored at 345 nm. After 35 min the cuvet was opened to the atmosphere and the disappearance of NADH was again monitored at 345 nm.

Under certain conditions the reduction of this enzyme by $NaBH_4$ can be shown to be UMP or substrate-dependent [3, 4], which indicates that uridine nucleotides increase the reactivity of epimerase–NAD^+ toward $NaBH_4$. This interpretation is made ambiguous by the fact that uridine nucleotides stabilize the epimerase–NADH complex against autoxidation [1, 2 ,4, 6], because it is equally possible that the sole function of uridine nucleotides in apparently promoting the reaction is to stabilize epimerase–NADH toward autoxidation [4]. The question can in principle be resolved by rate measurements, however, $NaBH_4$ is unstable in the near physiological pH range, making reliable rate measurements difficult. We find that $NaBH_3CN$ reduces the *E. coli* UDPgalactose 4-epimerase in a UMP-dependent reaction which is conveniently carried out under pseudo-first order conditions and monitored by the increase in $A_{345\ nm}$ due to NADH. Fig. 2 shows that the reaction is absolutely dependent

upon the presence of UMP under either aerobic or anaerobic conditions. In these experiments UMP cannot be acting only to stabilize epimerase–NADH toward autoxidation; if it were the reaction would be UMP-independent under anaerobic conditions. We conclude that UMP markedly increases the rate at which epimerase–NAD^+ reacts with $NaBH_3CN$. This fact constitutes chemical evidence that the binding of UMP induces a conformational transition in the active center of this enzyme.

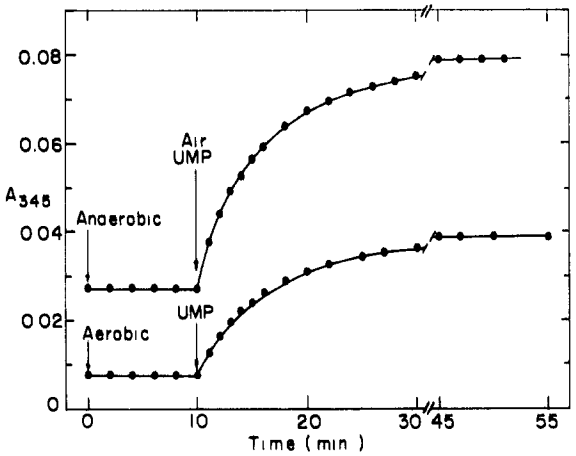

Fig. 2. UMP-dependent reduction of UDPgalactose 4-epimerase by $NaBH_3CN$. $NaBH_3CN$ from Ventron Chemical Corp. was recrystallized and analyzed titrimetrically by published procedures [7, 8] and found to be 92–94 % pure. The complete reaction mixture for the lower reaction progress curve contained 0.1 M Tris–HCl buffer (pH 8.5), 0.05 M $NaBH_3CN$, 3.0 mM UMP, 5.1 μM UDP-galactose 4-epimerase and the ionic strength was adjusted to 0.20 M with KCl. At zero time the reaction mixture was prepared minus UMP and the $A_{345\ nm}$ was monitored for 10 min before adding UMP. The reaction mixture for the upper curve was identical except for the enzyme concentration which was 8.4 μM. The solutions minus UMP were made anaerobic as described in Fig. 1 and mixed at zero time. After monitoring the $A_{345\ nm}$ for 10 min anaerobically the cuvet was opened to the atmosphere and the reaction was started by adding UMP. The half-time for the upper curve was 4.8 min and that for the lower curve was 5.0 min.

Data on the effect of $NaBH_3CN$ concentration on the observed pseudo-first order rate constants at 5.0 mM UMP are presented in Fig. 3. The reaction exhibits saturation kinetics with respect to $NaBH_3CN$, the apparent K_m for $NaBH_3CN$ being 0.051 M under the conditions of Fig. 3.

Data on the effect of UMP concentration on the observed first order rate constants at 0.05 M $NaBH_3CN$ are plotted in double reciprocal form in Fig. 4. The plot is curvilinear at large UMP concentrations, indicating complex kinetics above about 5 mM, however, at the smaller UMP concentrations the data approximate a straight line. The apparent K_m for UMP calculated from the extrapolated straight line in Fig. 4 is $1.6 \cdot 10^{-3}$ M, which is in reasonably good agreement with the inhibition constant $1.2 \cdot 10^{-3}$ M for UMP acting as a competitive reversible inhibitor (Frey, P. A., unpublished experiments). These parameters apply only to the reaction pathways which dominate the rate at small UMP concentrations. The curvature in Fig. 4 indicates that additional reaction pathways become important at large UMP concen-

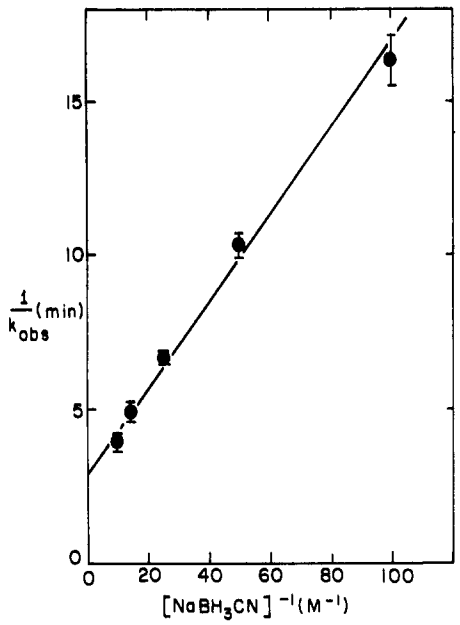

Fig. 3. The $NaBH_3CN$ concentration dependence for UMP-dependent reduction of the UDPgalactose 4-epimerase–NAD^+ complex. The rates were measured as described in Fig. 2. The reaction mixtures contained 0.1 M Tris–HCl buffer (pH 8.5), 5.0 mM UMP, 5.4 to 8.6 μM *E. coli* UDPgalactose 4-epimerase, $NaBH_3CN$ at the indicated concentrations, and sufficient KCl to maintain the ionic strength at 0.2 M in a total volume of 1.0 ml at 27 °C. Data points were taken from continuous chart records of the reaction progress, and the observed first order rate constants were evaluated graphically from those data. The points are mean values from 4 to 6 rate measurements and the error flags are average deviation from the mean.

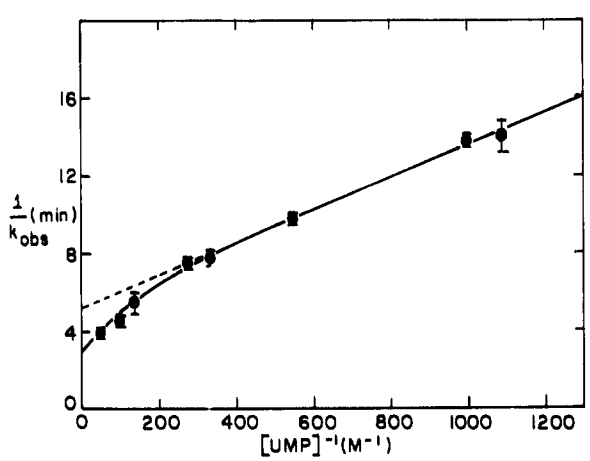

Fig. 4. The UMP concentration dependence for reduction by $NaBH_3CN$. The rates were measured as described in Fig. 3, except that the $NaBH_3CN$ concentration was constant at 0.050 M while the UMP concentration was varied as indicated.

trations. We have not studied this in detail, however, Plowman discusses downward curvature in such plots and also the various reaction patterns which produce it [9].

Free NAD^+ is itself reduced slowly by $NaBH_3CN$, so we can estimate the relative reactivities of NAD^+ and UMP-activated epimerase–NAD^+ toward this reagent. The comparison will be imperfect because borohydrides reduce free NAD^+ to a mixture of 1,2-; 1,4-; and 1,6-NADH, whereas $NaBH_4$ reduces the NAD^+ in epimerase–NAD^+ stereospecifically at C-4 and from the β-face [2, 4].

We measured the second order rate constant for the reaction of $NaBH_3CN$ with NAD^+ by making initial rate measurements, less than 5% of complete reaction, at five $NaBH_3CN$ concentrations between 0.01 and 0.18 M and five NAD^+ concentrations between 0.5 and 10 mM at pH 8.5, 27 °C and ionic strength 0.2 M adjusted with KCl. The rates were measured spectrophotometrically at 340 nm and calculated using the extinction coefficient $6.0 \cdot 10^3\ M^{-1} \cdot cm^{-1}$, which we measured by reducing a known concentration of NAD^+ to the product mixture with $NaBH_3CN$. The initial rates were proportional to the first power of the concentrations of both reactants, and the mean of 27 calculated rate constants was $0.0631 \pm 0.0057\ M^{-1} \cdot min^{-1}$, with no apparent tendency for the calculated constants to drift upward or downward with increasing concentrations of either reactant.

We cannot compare rate constants for enzyme–NAD^+ complex and free NAD^+ directly because the reaction with the enzyme follows saturation rather than second order kinetics, however, we can make indirect comparisons as follows: The apparent maximum rate constant in Fig. 3 is $0.357\ min^{-1}$. From the apparent K_m for UMP in Fig. 4 we estimate the UMP concentration in Fig. 3 to be about 80% of saturation, so that at saturation the maximum rate constant would be about $0.45\ min^{-1}$. The apparent maximum rate constant from the linear portion of Fig. 4 is $0.192\ min^{-1}$. Since the $NaBH_3CN$ concentration in Fig. 4 is about the apparent K_m for $NaBH_3CN$ in Fig. 3, the actual maximum rate would be about $0.39\ min^{-1}$. The average of these two values, $0.42\ min^{-1}$, should be a conservative estimate for the maximum rate constant corresponding to the dominant reaction pathways at low UMP concentrations. If the curvature in Fig. 4 is taken into account we can estimate the maximum rate constant for the dominant pathways at high UMP concentrations at $0.72\ min^{-1}$. When these estimated rate constants are divided by $0.0631\ M^{-1} \cdot min^{-1}$ the ratios are 6.7 M and 11.4 M, respectively, which means that the "effective concentration" of BH_3CN^- at the active site is about 6.7 to 11.4 M, or that, at a given equal concentration of epimerase–NAD^+ and free NAD^+, 6.7 to 11.4 M $NaBH_3CN$ is required to reduce NAD^+ at the same rate as epimerase–NAD^+ when UMP is saturating.

Alternatively we can compare the second order rate constant for NAD^+ with the apparent second order rate constant for epimerase–NAD^+ calculated from the apparent K_m for $NABH_3CN$ and maximum rate. These constants are $0.0631\ M^{-1} \cdot min^{-1}$ and $8.4\ M^{-1} \cdot min^{-1}$ respectively, which shows that under second order conditions, i.e., $NaBH_3CN$ concentrations well below K_m, the rate of the UMP activated reaction of epimerase–NAD^+ is about 130 times that for an equivalent concentration of NAD^+.

By either comparison the UMP activated reaction of $NaBH_3CN$ with epimerase–NAD^+ is substantially faster than that with NAD^+ itself, at least at low $NaBH_3CN$ concentrations. The reaction with the enzyme exhibits saturation kinetics,

which indicates that a binding step precedes the actual reduction, and the binding process probably accounts, at least in part, for the increased rate.

ACKNOWLEDGMENT

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