Dehydroquinate Synthase: The Role of Divalent Metal Cations and of Nicotinamide Adenine Dinucleotide in Catalysis

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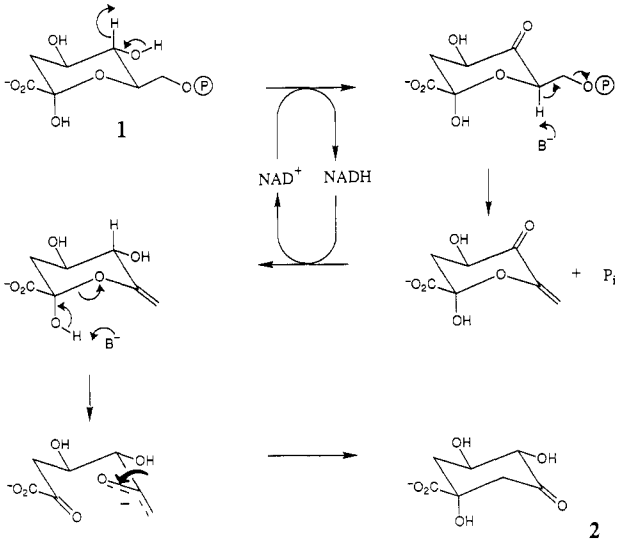
ABSTRACT: The cofactor requirements of dehydroquinate synthase from *Escherichia coli* have been characterized. The homogeneous enzyme, purified from the overproducing strain RB791 (pJB14), is a monomeric metalloenzyme of M_r = 39000 that contains 1 mol of tightly bound Co(II) according to atomic absorption analysis. The holoenzyme rapidly loses activity upon incubation with EDTA, giving rise to a stable but catalytically inactive apoenzyme. Activity is fully restored by reconstitution with Co(II) and partially restored with other divalent cations. Reconstitution of the apoenzyme with Zn(II) (which is probably the functioning metal in vivo) restores activity to 53% of the level observed with the Co(II)–holoenzyme. The presence of the substrate 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (**1**) blocks the inactivation by EDTA. Dehydroquinate synthase also binds 1 mol of NAD^+, the presence of which is essential for catalytic activity. The rate constant for the dissociation of NAD^+ from the Co(II)–holoenzyme was found to be 0.024 min^{-1}. Under turnover conditions with saturating levels of substrate, the dissociation rate of NAD^+ increases by a factor of 40, to 1 min^{-1}. Under these conditions (pH 7.5, 20 °C), the K_m for NAD^+ was determined to be 80 nM.

In plants and microorganisms, the enzymes of the shikimate pathway are responsible for the biosynthesis of the three aromatic amino acids and a range of other primary and secondary metabolites (Haslam, 1974; Weiss & Edwards, 1980). The second enzyme in the pathway, dehydroquinate synthase, catalyzes the conversion of 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP,[1] **1**) to dehydroquinate (DHQ, **2**) (Srinivasan et al., 1963). A variety of mechanistic studies including the determination of the overall stereochemical course of the reaction (Rotenberg & Sprinson, 1970; Turner et al., 1975), the measurement of primary tritium kinetic isotope effects (Rotenberg & Sprinson, 1978; Le Marêchal & Azerad, 1976a), and the use of substrate analogues (Le Marêchal & Azerad, 1976b; Le Marêchal et al., 1980; Widlanski et al., 1987) have led to the proposed mechanistic pathway shown in Scheme I (Srinivasan et al., 1963; Maitra & Sprinson, 1978). In this sequence, which is mechanistically unusually diverse for a single enzyme, the synthase appears to mediate five transformations: (i) the oxidation of the secondary alcohol at C-5; (ii) the β-elimination of inorganic phosphate across C-6 and C-7; (iii) the reduction of the resulting enone at C-5; (iv) the ring opening of the enol pyranose; (v) the final intramolecular aldol-like reaction that produces DHQ.

Before embarking on a detailed investigation of this multistep enzymic reaction, we first constructed a strain of *Escherichia coli* that overproduces dehydroquinate synthase by more than 1000-fold (Frost et al., 1984) to avoid the experimental limitation imposed by the low abundance of the enzyme in wild-type *E. coli* cells. We and others have reported synthetic routes to the substrate DAHP (Srinivasan et al., 1963; Trigalo et al., 1975; Herrmann & Polig, 1975; Aldersberg & Sprinson, 1984; Frost & Knowles, 1984), as well as an efficient procedure for the isolation of this material from the supernatant of the growth medium of an appropriately blocked *E. coli* mutant (Frost & Knowles, 1984). In this and the following two papers, we report our investigations on the nature and mechanistic detail of the enzyme-catalyzed reaction. In this first paper we define the cofactor requirements and the kinetic behavior of the homogeneous enzyme. In the following two papers are described the results of studies with substrate analogues designed to illuminate each step of the enzyme reaction mechanism.

Scheme I: Proposed Mechanistic Scheme for the Enzyme-Catalyzed Conversion of 3-Deoxy-D-*arabino*-heptulosonate 7-Phosphate (**1**) to Dehydroquinate (**2**)



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[1] Abbreviations: ADPR, adenosine diphosphoribose; DAHP; 3-deoxy-D-*arabino*-heptulosonate 7-phosphate; DEAE, diethylaminoethyl; DHQ, dehydroquinate; EDTA, ethylenediaminetetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonate; MES, 2-(*N*-morpholino)ethanesulfonate; MOPS, 3-(*N*-morpholino)propanesulfonate; NAD^+, nicotinamide adenine dinucleotide; NADH, the reduced form of NAD^+.

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Experimental Procedures

Materials

DAHP was isolated from the growth medium of the auxotrophic strain *E. coli* JB-5, as previously described (Frost & Knowles, 1984). Dehydroquinate synthase was isolated from *E. coli* RB791 (pJB14) as described earlier (Frost et al., 1984). Dehydroquinase was purified from the overproducing strain AB2848 (pKD201) according to the procedure of Coggins and his collaborators (Duncan et al., 1986), through the DEAE-Sephacel step, to material having a specific catalytic activity of 175 units/mg. High-purity metal salts were either of Gold Label grade from Aldrich (Milwaukee, WI) or of Puratronic grade from Johnson-Matthey (Seabrook, NH). High-purity NAD^+ (grade I) was obtained from Boehringer Mannheim (Indianapolis, IN). Adenosine 5′-diphosphoribose was from Sigma (St. Louis, MO).

Methods

Spectrophotometric measurements were made on a Hewlett-Packard 8452A diode array spectrophotometer (for kinetic measurements) or on a Perkin-Elmer 555 spectrophotometer (for other absorbance measurements). Atomic absorption analysis as conducted by Dr. Robert Shapiro and David Evenson at Harvard Medical School, using a Perkin-Elmer 5000 electrothermal atomic absorption spectrophotometer.

Assays. Enzyme assay solutions (1.0 mL) were made up in deionized, glass-distilled water containing 50 mM MOPS buffer, pH 7.75, cobalt(II) sulfate (50 μM), NAD^+ (10 μM), DAHP (0.50 mM), and dehydroquinase (1 unit). After equilibration at 20 °C for 5 min, dehydroquinate synthase (10–50 milliunits) was added, and the production of dehydroshikimate was monitored spectrophotometrically at 234 nm (Mitsuhashi & Davis, 1954; Maitra & Sprinson, 1978). The initial rate was calculated from a linear least-squares fit of the first 30 s of the progress curve. The effective extinction coefficient for the coupled assay under these conditions was 9850 M^{-1} cm^{-1}. This value was determined experimentally by measurement of the change in absorbance at 234 nm under the conditions described, when DHQ synthase and dehydroquinase were added to a stock solution of DAHP of known concentration that had been determined by quantitative assay of the P_i released either by DHQ synthase or by alkaline phosphatase. One unit of enzyme activity catalyzes the consumption of 1 μmol of DAHP/min at 20 °C.

For the determination of the steady-state kinetic parameters, rate measurements were made as described above, except that the pH was 7.5, the cobalt(II) sulfate concentration was 20 μM, and the amount of dehydroquinate synthase used was reduced to 1 milliunit. A 5-s delay was allowed after initiation of the reaction. The initial rate was then determined by least-squares fitting of the first 5% of the progress curve (between 30 and 90 s, depending on the initial concentration of DAHP) to a straight line. This rate was verified by following the progress curve with time as described under Data Analysis.

Metal Ion Studies. Divalent metal ions were removed from solutions of DAHP and dehydroquinase by stirring with Chelex resin, followed by filtration through a Millipore 0.22-μm sterilizing membrane. Water and buffers were passed through a column of Chelex and stored in EDTA-washed polyethylene bottles.

(*a*) *Atomic Absorption Analysis.* Dehydroquinate synthase [6 mg, in 1 mL of 50 mM sodium phosphate buffer, pH 6.6, containing cobalt(II) sulfate (100 μM) and NAD^+ (20 μM)] was dialyzed overnight against 2 × 1 L of 50 mM MOPS buffer pH 7.5, containing NAD^+ (20 μM). To the second portion of dialyzing buffer, Chelex resin (2 mL) was added. The concentration of enzyme was determined to be 72 μM by Bradford assay (Bradford, 1976). Atomic absorption analysis for cobalt gave a concentration of 71 μM.

(*b*) *Preparation of the Apoenzyme.* To 50 mM MOPS buffer, pH 7.5, containing NAD^+ (10 μM) and EDTA (200 μM) (14 mL) was added dehydroquinate synthase (6 mg) in 50 mM sodium phosphate buffer containing cobalt(II) sulfate (100 μM) and NAD^+ (20 μM) (1 mL). The solution was concentrated to 1 mL by ultrafiltration and then diluted with 50 mM metal-free MOPS buffer containing NAD^+ (10 μM) (14 mL). The resulting solution was concentrated to approximately 1 mL to give apodehydroquinate synthase (approximately 100 μM). Assay of this solution in the presence of EDTA (100 μM) gave <1% the rate of an assay performed in the presence of cobalt(II) sulfate (50 μM).

(*c*) *Determination of the Rate of Metal Ion Loss in the Presence of EDTA.* In an assay cuvette at 20 °C, cobalt(II)-containing DHQ synthase (20 μL of a solution of 125 μM) was added to 50 mM MOPS buffer, pH 7.5, containing NAD^+ (10 μM), dehydroquinase (1 unit), and EDTA (50, 100, or 250 μM), in a total volume of 0.96 mL. After a measured period of time, the reaction was initiated by the addition of DAHP (20 μL of a solution of 14 mM), and the initial rate was determined within 30 s. The rates at each concentration of EDTA were fitted to an exponential expression by using a nonlinear least-squares fit to obtain the pseudo-first-order rate constant for the loss of catalytic activity.

(*d*) *Determination of the Rate of Loss of Co(II) by Exchange with Cd(II).* A sample of Co(II)–DHQ synthase (10 μL of a solution of 40 μM) was added to 50 mM MOPS buffer, pH 7.5, containing cadmium(II) sulfate (0.50 mM) (0.99 mL) at 20 °C. A timed intervals, portions (20 μL) were assayed for catalytic activity as described above, except that no Co(II) was present in the assay buffer. The experimentally determined rates were fitted to an equation containing an exponential term plus a constant to obtain the rate constant for the dissociation of Co(II) from the enzyme.

(*e*) *Reconstitution Experiments.* To a portion (0.49 mL) of 50 mM MOPS buffer, pH 7.5, containing ultrapure divalent metal ion (50 μM) was added apodehydroquinate synthase (10 μL of a solution of 100 μM). A portion (10 or 20 μL) of this solution was assayed for activity in 50 mM MOPS buffer (1.0 mL), pH 7.75, containing DAHP (0.3 mM), EDTA (0.2 mM), NAD^+ (10 μM), and dehydroquinase (1 unit).

NAD^+ Studies. (*a*) *Determination of Bound NAD^+.* A solution of dehydroquinate synthase (7.7 mg, 0.20 μmol) in 100 mM MOPS buffer, pH 7.4 (1 mL), was dialyzed against 3 × 600 mL of NAD^+-free 25 mM MOPS buffer, pH 7.4, over 3 days. Portions (150 μL) were removed before each buffer change, of which a fraction (10 μL) was employed for determining the protein concentration by Lowry analysis (Lowry et al., 1951). To the remaining 140 μM was added ethanol (50 μL), and the mixture was warmed at 40 °C for 2 min. Denatured protein was removed by centrifugation, and the supernatant was diluted to 0.50 mL with 70 mM sodium pyrophosphate buffer containing semicarbazide hydrochloride. Yeast alcohol dehydrogenase (2 μL, approximately 10 units) was added, and the concentration of NAD^+ was calculated from the absorbance change at 340 nm due to the formation of NADH (Bergmeyer, 1984).

(*b*) *Determination of k_{off} for NAD^+.* (*i*) *In the Absence of Substrate.* To a solution of 50 mM MOPS buffer, pH 7.5, containing cobalt(II) sulfate (50 μM) and adenosine 5′-di-

phosphoribose (ADPR) (50 μM) (0.38 mL) at 20 °C was added dehydroquinate synthase (20 μL of a solution of 25 μM). At timed intervals, portions (10 μL) were removed and assayed for catalytic activity. The first-order rate constant for NAD^+ loss was determined by fitting the experimental rates to an exponential expression. In the control mixture, ADPR was replaced by NAD^+.

(*ii*) *During Turnover.* A portion of the enzyme solution after dialysis against NAD^+-free MOPS buffer, pH 7.5, was assayed under the usual conditions except that ADPR (10 μM) replaced NAD^+ in the assay solution. The first-order rate constant for loss of catalytic activity was obtained by a nonlinear least-squares fit of the progress curve to an exponential expression. Omission of ADPR from the assay buffer did not affect the initial exponential decay but resulted in a small residual linear rate at the steady state. After this steady state had been reached, addition of NAD^+ (5 μM) resulted in the reactivation of the enzyme to the same activity as that obtained in a normal assay with NAD^+.

(*c*) *Determination of K_m for NAD^+.* Assays were conducted as usual except that the NAD^+ concentration was varied between 25 nM and 5 μM. The rates were obtained from a linear fit of the progress curve at 200–300 s after mixing (to allow for the establishment of the steady state with respect to NAD^+ binding). The K_m for NAD^+ was determined by fitting the experimental rates to the Michaelis–Menten equation.

Reactivation of Deactivated Dehydroquinate Synthase. To 50 mM MOPS buffer, pH 7.0, containing NAD^+ (8 μM), cobalt(II) sulfate (50 μM), DAHP (1.1 mM), and dehydroquinase (2.2 unit) (in a total volume of 2.5 mL) at 30 °C was added apodehydroquinate synthase (1 μL of a solution of 125 μM). The absorbance at 270 nm ($\Delta\epsilon$ = 2200 M^{-1} cm^{-1}) was monitored until the linear region of the progress curve was reached (13 min). The ratio of the final velocity to the initial velocity was approximately 0.1. The solution was concentrated by ultrafiltration to 0.1 mL in a Centricon filter, then 50 mM MOPS, pH 7.5, containing EDTA (50 μM) (1 mL) was added, and the solution was concentrated to 350 μL. After the solution was warmed to 20 °C, portions (20 μL) were assayed [in the presence of cobalt(II) sulfate (50 μM)] at timed intervals. The control incubation was processed identically except that DAHP was absent from the original solution.

Data Analysis. Velocities were obtained and fitted to the appropriate kinetic equation by using the kinetics software package on the Hewlett-Packard UV–vis ChemStation. In the determination of K_m (DAHP), velocities at DAHP concentrations below 30 μM were routinely obtained from the progress curve as follows. The end-point absorbance was used to determine the absorbance values along the progress curve at which a specified concentration of DAHP remained. The rate at this concentration was then obtained by a linear least-squares fit of a small region of the progress curve in which the appropriate absorbance value was centered. The Michaelis constants obtained in this manner were within experimental error of those obtained solely from initial velocities.

Results

Enzyme Properties. In their original work on dehydroquinate synthase from *E. coli*, Sprinson and co-workers (Srinivasan et al., 1963; Maitra & Sprinson, 1978) found that the partially purified enzyme requires catalytic levels of NAD^+ and Co(II) for both activity and stability. Our initial studies of the homogeneous enzyme therefore employed both of these cofactors in all the buffers used. In the presence of Co(II) and NAD^+ at pH 7.5 in 100 mM MOPS buffer, the free

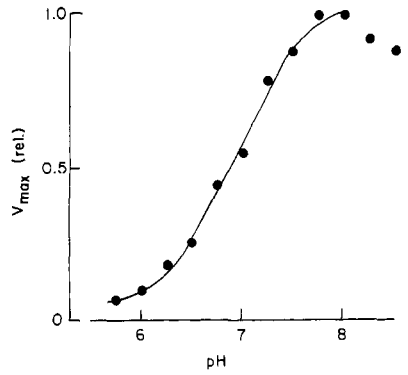

FIGURE 1: pH dependence of V_{max} for the reaction catalyzed by dehydroquinate synthase. The concentration of DAHP was 700 μM in one of the following buffers (50 mM), containing NAD^+ (200 μM) and cobalt(II) sulfate (50 μM): MES (pH 5.75–6.75); MOPS (pH 6.5–8.0); or HEPES (pH 7.75–8.5).

enzyme is stable for several months at 4 °C. The kinetic constants (k_{cat} and K_m) determined from initial velocity studies with DAHP as substrate indicate that DHQ synthase is a very efficient catalyst, with a value of k_{cat}/K_m of 2.5 × 10^7 M^{-1} s^{-1} at 20 °C and pH 7.75. The pH dependence of V_{max} suggests the presence of a catalytically important ionization with an apparent pK_a of about 7.0 (Figure 1). The value of K_m for DAHP also increases with pH, from 0.6 μM at pH 6.5 to 5.5 μM at pH 8.5. The K_m for DAHP has been determined in three other laboratories to be 50 (Le Marêchal & Azerad, 1976a), 35 (Maitra & Sprinson, 1978), and 19 μM (Myrvold et al., 1989). These values were determined under conditions somewhat different from those used in the present work. More importantly, however, we have found that the observed value of K_m depends upon the assay method used. The P_i release assay is relatively insensitive and requires the use of enzyme concentrations that give a rather rapid substrate consumption. This method leads inevitably to an overestimation of the K_m value because at low initial substrate concentrations the early part of the progress curve is difficult to monitor. This problem can be overcome both by using the whole progress curve to estimate the initial rate and by slowing the reaction down by using less enzyme. In our early work we had avoided using low enzyme concentrations because of the apparent time-dependent inactivation of the synthase. However, since the discovery of the origin of the inactivation (see below) and the routine use of highly purified nicotinamide cofactor, the determination of initial rates at substrate concentrations as low as 5 μM become routine, and precise initial rate measurements become possible.

Divalent Metal Ion Requirement. Dialysis of the purified enzyme against metal-free buffer provides a fully active Co(II)–enzyme complex of 1:1 stoichiometry as determined by atomic absorption analysis. Treatment of the Co(II)–holoenzyme with EDTA in the absence of substrate results in the rapid loss of catalytic activity. The rate of activity loss is first order with respect to EDTA concentration (and has a second-order rate constant of 10^4 M^{-1} s^{-1}), which indicates that EDTA actively participates in metal ion loss rather than simply sequesters free Co(II) after it has dissociated from the enzyme. In the presence of saturating levels of the substrate DAHP, the time course for product formation is essentially unaffected by the presence of EDTA, which suggests that the metal is less accessible to EDTA when the substrate is bound to the active site.

Addition of Co(II) to the apoenzyme restores full catalytic activity. Other divalent metals also support the reaction to varying degrees (Table I). The activities in Table I were

Table I: Catalytic Activity of Dehydroquinate Synthase Reconstituted with Various Metal Cations[a]

divalent ion	relative V_{max}	divalent ion	relative V_{max}
Co	100	Cu	9
Zn	53	Mg	0
Ni	23	Ca	0
Cd	12	Sr	0
Mn	10	Ba	0

[a] For conditions of reconstitution, see Experimental Procedures.

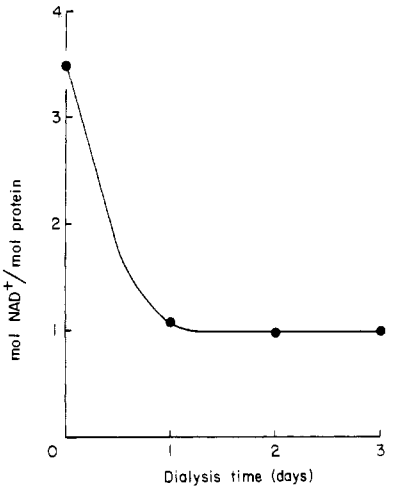

FIGURE 2: Mole ratio of NAD^+ to dehydroquinate synthase as a function of dialysis time. Enzyme that had been preincubated with [^{14}C]NAD^+ was then dialyzed against successive changes of buffer to which no NAD^+ had been added.

determined by reconstituting the apoenzyme (0.5 μM) with a 100-fold molar excess of high-purity divalent metal ion and then assaying in the presence of a molar excess of EDTA over metal. If the apoenzyme was directly assayed in the presence of the various metal ions, small but variable rates were observed for the "nonactivating" metals. We attribute this behavior to contamination of the assay buffers with very low levels of active metal ions that the enzyme can utilize. Since the presence of substrate effectively blocks metal loss from the enzyme, the problem of adventitious metal ions can thus be avoided.

Under the assay conditions described that contain EDTA in the assay buffer, V_{max} for the Zn(II)–holoenzyme is about half that of the Co(II)–holoenzyme. The presence of excess Zn(II) in the assay buffer leads, however, to decreased rates showing mixed-type inhibition kinetics, which suggests that zinc may also bind to a second, lower affinity inhibitory site. The inhibition we observe at high Zn(II) levels may explain the results of previous workers (Maitra & Sprinson, 1978) who have been unsuccessful in reconstituting the apoenzyme with relatively high concentrations of Zn(II). Furthermore, the Zn(II)–holoenzyme loses activity rapidly ($t_{1/2} \sim 30$ min), which is accompanied by the eventual precipitation of protein. In contrast to this behavior, both the Co(II)–holoenzyme and the apoenzyme are stable for months at 4 °C, and an excess of Co(II) does not inhibit the Co(II)-activated enzyme.

NAD^+ Requirement. Dialysis of purified enzyme that has been stored in the presence of Co(II) and NAD^+ against NAD^+-free buffer allows the preparation of a stoichiometric, fully active enzyme·NAD^+·Co(II) complex (see Figure 2). Incubation of the stoichiometric enzyme·NAD^+·Co(II) complex with adenosine diphosphoribose (ADPR) and periodic removal of portions for assay of the remaining catalytic activity give a rate constant for activity loss of 4×10^{-4} s^{-1} that evidently derives from the departure of NAD^+ from the enzyme. In contrast to this behavior in the absence of substrate, when NAD^+ in the standard assay buffer is replaced by adenosine diphosphoribose (ADPR), the rate of product formation decreases exponentially with time, with a first-order rate constant of 0.017 s^{-1} at 20 °C and pH 7.5 (Figure 3). If neither NAD^+ nor ADPR is added to the assay buffer, the exponential time course is similar except that a small linear rate persists (see Figure 3). This final steady-state reaction can be ascribed to the equilibrium amount of functional enzyme-NAD^+ complex that is present even at the low concentrations of enzyme and NAD^+ in the solution. Addition of NAD^+ to the solution increases this final rate, and from these increases the K_m for NAD^+ is found to be 80 nM (pH 7.75, 20 °C). From this value and the value of k_{off} measured as described above, k_{on} is calculated to be 2×10^5 M^{-1} s^{-1}. As also shown in Figure 3, after the steady state at the reduced rate has been achieved, full enzyme activity can be recovered by the addition of NAD^+ to micromolar levels. The apoenzyme (that lacks NAD^+) appears to be reasonably stable, since reactivation by NAD^+ can be effected even after 30 min of the slow steady-state reaction.

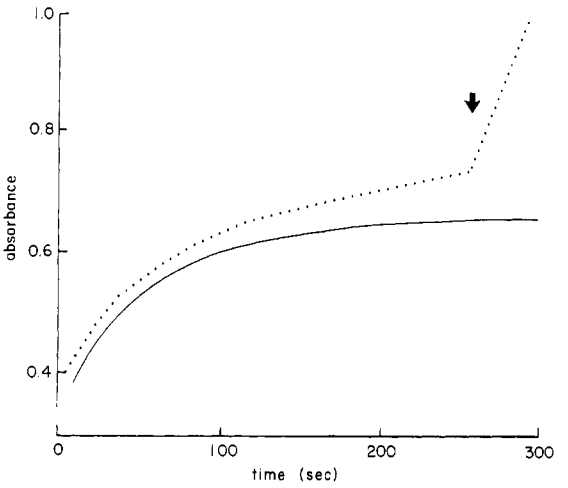

FIGURE 3: Progress curves for the reaction catalyzed by dehydroquinate synthase under conditions in which NAD^+ dissociates from the enzyme. The dotted curve was obtained by addition of the stoichiometric NAD^+–enzyme complex to the standard assay buffer lacking NAD^+. At the time shown by the arrow, NAD^+ was added to bring the concentration of NAD^+ to 20 μM. The solid curve was obtained by addition of the stoichiometric NAD^+–enzyme complex to the standard assay buffer containing ADPR (20 μM) in place of NAD^+.

From the above results, we see that the nicotinamide cofactor dissociates from the enzyme more rapidly in the presence of substrate. These observations are consistent with the loss of NAD^+ either from the Michaelis complex or from one of the later intermediate complexes of the catalytic cycle in a reaction that is slow relative to turnover (k_{cat}/k_{off} is $\sim$3000) and that results in the formation of inactive apoenzyme. Since it was possible that enzyme inactivation during turnover was due to the dissociation of NADH rather than of NAD^+ (which could leave the enzyme blocked by oxidized substrate), the identity of the released cofactor was established by submitting enzyme containing [U-^{14}C]NAD^+ to the usual turnover conditions for 5 min. The free cofactor was isolated by gel filtration on Bio-Gel P-4, and the identity of the liberated nicotinamide derivative was established by ion-exchange chromatography. This analysis showed that the released cofactor was >95% NAD^+, not NADH. These observations relate to the Co(II)–enzyme. With the Zn(II)–enzyme, the dissociation rate of NAD^+ is slower by a factor of about 10 and is slightly

dependent on the total concentration of Zn(II), being somewhat faster at 25 μM Zn(II) than at 300 μM Zn(II).

Discussion

In this paper we describe the nature of the reaction catalyzed by dehydroquinate synthase and define the cofactor requirements of this interesting enzyme. As isolated by the existing purification protocol, dehydroquinate synthase contains a precisely stoichiometric amount of tightly bound Co(II) that is essential for catalytic activity. Treatment of the isolated metalloenzyme with EDTA results in the rapid formation of inactive apoenzyme, that may be reconstituted by the addition of a variety of different divalent metal cations. The identity of the metal ion not only influences the catalytic activity of the enzyme but also affects the rate of NAD^+ dissociation from the active site during turnover. Moreover, substrate binding prevents the removal of Co(II) by EDTA. Taken together, these results demonstrate a central role for the metal ion in the catalytic mechanism. Indeed, we should not be surprised by the involvement of a metal, since *each* of the four types of chemical transformation mediated by dehydroquinate synthase has, in simpler enzymic systems, been shown to involve metal cofactors. Thus, the involvement of divalent metal cations in NAD^+-dependent redox reactions [e.g., Bertini et al. (1983) and Brändén and Eklund (1978)], in β-elimination processes where water or phosphate ion is lost [e.g., Shen and Westhead (1973)], in pyranose ring opening (Rose et al., 1973), and in aldol reactions [e.g., Kobes et al. (1969) and Kadonaga and Knowles (1983)] is well precedented in enzymology. Access to a variety of metal-substituted holoenzymes now permits the design of many experiments in which the metal ion serves as a probe of events at the active site. It is anticipated that these experiments will shed additional light on the role of the metal ion in the mechanism of dehydroquinate synthase.

On the basis of the greater bioavailability of Zn(II), it seems likely that dehydroquinate synthase is naturally a Zn(II)–metalloenzyme as proposed by Lambert et al. (1985). Yet for in vitro studies, Co(II) offers several practical advantages, and the similarity of k_{cat} and K_m for the Co(II)– and Zn(II)–enzymes suggests that no major changes in the nature or mechanism of catalysis occur when one metal is substituted for the other. Subtle differences in active-site interactions for the two metals are evident, however, some of which are discussed below.

In the proposed mechanism (see Scheme I), NAD^+ is employed catalytically to effect a transient oxidation at C-5 of the substrate, and the redox cofactor need never dissociate from the active site. The observed tight binding of NAD^+ to the free enzyme is therefore not surprising. It is less easy, however, to rationalize the 50-fold increase in the rate of NAD^+ dissociation from the Co(II)–enzyme when substrate is present. Significantly, NAD^+ dissociates much more slowly from the Zn(II)–enzyme [than from the Co(II)–enzyme] during turnover, which may better reflect the in vivo situation. In addition, the second, inhibitory [Zn(II)–selective] metal binding site also slows the rate of NAD^+ release somewhat. Finally, the pH dependence of the NAD^+ off-rate may derive simply from the titration of a base involved in the binding interaction or, possibly, from a change in the steady-state population of the particular complex from which NAD^+ dissociates.

From a practical viewpoint, the substrate-induced dissociation of NAD^+ from the Co(II)–enzyme is not as benign as it may appear. As shown by the experiments with ADPR, the apoenzyme that results from this process can bind NAD^+ analogues. Furthermore, it appears that, even in the presence of DAHP, such analogues bind with great affinity, similar to the binding of NAD^+ to the free enzyme. Thus, turnover conditions create a special opportunity for inhibition by NAD^+ analogues, such that the NAD^+ employed in the assay buffer must be of extremely high purity. The use of samples of NAD^+ that are not of the highest purity results in the exponential loss of catalytic activity to a new steady state in which much of the enzyme has bound some unidentified inhibitor.

There exists a relatively small group of enzymes [recently reviewed by Frey (1987)] that require a nicotinamide cofactor even though the overall catalyzed transformation is redox neutral. Each member of this group actually exploits the bound cofactor in an oxidative activation step, the substrate being reduced again later in the reaction sequence. In this sense, the group of enzymes differs from those (such as glycogen phosphorylase or chorismate synthase) that contain a seemingly gratuitous cofactor, the function of which (in effecting the catalyzed reaction, at least) is obscure. The behavior of the nicotinamide cofactor in dehydroquinate synthase, in contrast, parallels the properties of NAD^+ bound to other enzymes in this class [e.g., myoinositol synthase or UDPglucose epimerase (Frey, 1987)]. Indeed, as will become apparent from the results reported in the following two papers, dehydroquinate synthase may need to be little more than an internally cycling dehydrogenase, for which purpose a single NAD^+ and a single Zn(II) are unsurprising adjuvants.

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Dehydroquinate Synthase: The Use of Substrate Analogues To Probe the Early Steps of the Catalyzed Reaction†

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ABSTRACT: The early steps of the proposed mechanistic pathway for dehydroquinate synthase have been probed with a series of substrate analogues. These analogues, **3**–**9**, are structurally prohibited from undergoing the β-elimination of inorganic phosphate that represents the committed step in the conversion of the substrate 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (**1**) to dehydroquinate (**2**). In agreement with previous observations, the analogues that possess shortened side chains (**3**, **5**, and **6**) bind more tightly to the enzyme than those (**4** and **7**–**9**) that are more nearly isosteric with the substrate. Two hitherto unrecognized factors that influence binding have been identified: (i) carbacyclic analogues bind 25–100 times more tightly than the corresponding oxacyclic materials (indeed, the carbacyclic phosphonate **5** has a K_i value of 8×10^{-10} M) and (ii) the side chain appears to be bound in a gauche conformation similar to the most stable conformation of the *cis*-vinylhomophosphonate **8**. These trends in binding can be rationalized by considering the behavior of the analogues in the first two chemical steps of the mechanism: NAD^+-mediated oxidation at C-5 and enolization at C-6 (the first part of the E1cB elimination of inorganic phosphate). Direct spectrophotometric determination of the equilibrium level of enzyme-bound NADH indicates that the carbacyclic analogues are more readily oxidized than the oxacyclic compounds, and this predictable difference in redox behavior is reflected in the observed differences in binding. The gauche conformation of the C-7 side chain appears to be required for proton abstraction from C-6, since only those analogues that can adopt this conformation undergo enzyme-catalyzed exchange of the C-6 proton with the solvent. This conformation positions one of the peripheral oxygens of the phosphate (or phosphonate) group close to the C-6 proton. Taken together with other data, these results suggest that the enzyme exploits this substrate base in the enolization, which occurs through an intramolecular proton transfer. The loss of P_i then completes the β-elimination.

In plants and microorganisms, aromatic amino acids are biosynthesized through the action of the enzymes of the shikimate pathway (Haslam, 1974; Weiss & Edwards, 1980). The second enzyme in the pathway, dehydroquinate synthase, catalyzes the conversion of 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP,[1] **1**) to dehydroquinate (DHQ, **2**) (Srinivasan et al., 1963).

In this and the following paper, a number of substrate analogues are described that are processed by dehydroquinate synthase to various stages along the reaction pathway illustrated in Scheme I. This approach allows the multistep mechanism to be dissected into its component transformations so that the existence of each step is verified and its mechanism is scrutinized. Furthermore, important characteristics of the enzyme mechanism become clear by examination of the trends observed for a given experimental parameter with a series of

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[1] Abbreviations: ADPR, adenosine diphosphoribose; DAH, 3-deoxy-D-*arabino*-heptulosonate; DAHP, 3-deoxy-D-*arabino*-heptulosonate 7-phosphate; DEAE, diethylaminoethyl; DHQ, dehydroquinate; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; MOPS, 3-(*N*-morpholino)propanesulfonate; NAD^+, nicotinamide adenine dinucleotide; NADH, reduced form of NAD^+; THF, tetrahydrofuran.

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