

Kinetic Isotope Effects on the Oxidation of Reduced Nicotinamide Adenine Dinucleotide Phosphate by the Flavoprotein Methylenetetrahydrofolate Reductase[†]

Maria A. Vanoni[‡] and Rowena G. Matthews*

ABSTRACT: Previous work from this laboratory has established that the NADPH–menadione oxidoreductase reaction catalyzed by methylenetetrahydrofolate reductase from pig liver proceeds by Ping Pong Bi Bi kinetics and that the reductive half-reaction is rate limiting in steady-state turnover. We have now shown that methylenetetrahydrofolate reductase stereospecifically removes the *pro-S* hydrogen from the 4-position of NADPH. During the oxidation of [4(*S*)-³H]NADPH, we observed a kinetic isotope on V/K_{NADPH} of 10.8 ± 0.4 . When comparing the rates of oxidation of [4(*S*)-²H]NADPH and [4(*S*)-¹H]NADPH, we measure kinetic isotope effects on V of 4.78 ± 0.15 and on V/K_{NADPH} of 4.54 ± 0.59 . When oxidation of [4(*R*)-²H]NADPH and [4(*R*)-¹H]NADPH is compared, the secondary kinetic isotope effect on V is 1.04 ± 0.01 . When the NADPH–menadione oxidoreductase reaction is catalyzed in tritiated water, no incorporation of solvent tritium into residual NADPH is observed. We conclude from these observations that the oxidation of NADPH is largely or entirely rate limiting in the reductive half-reaction and, hence, in NADPH–menadione oxidoreductase turnover

Methylenetetrahydrofolate reductase catalyzes the irreversible NADPH-linked reduction of $\text{CH}_2\text{-H}_4\text{folate}^1$ to $\text{CH}_3\text{-H}_4\text{folate}$. This reaction commits H_4folate -bound one-carbon units to the pathways of AdoMet-dependent biological methylations. Enzyme activity in mammalian cells is regulated by the allosteric effector AdoMet (Kutzbach & Stokstad, 1971; Vanoni et al., 1983b). Studies from this laboratory have established that the enzyme catalyzes the transfer of reducing equivalents from NADPH to electron acceptors such as $\text{CH}_2\text{-H}_4\text{folate}$ and menadione by Ping Pong Bi Bi mechanisms and that the reduction of the enzyme by NADPH is irreversible, while reoxidation of the reduced flavoprotein by $\text{CH}_2\text{-H}_4\text{folate}$ is freely reversible (Daubner & Matthews, 1982a; Vanoni et al., 1983a). In the presence of saturating menadione, the reductive half-reaction appears to be rate limiting in steady-state turnover (Vanoni et al., 1983a); i.e., it is the most sensitive half-reaction in the more accurate terminology introduced by Ray (1983). Thus, the enzyme catalyzes a thermodynamically irreversible physiological reaction where the irreversible segment of the overall reaction is amenable to study by means of steady-state kinetic measurements in the presence of the artificial electron acceptor menadione.

Albery & Knowles (1976) have proposed a series of criteria for the catalytic efficiency of an enzyme. In particular, they suggested that diffusion of physiological concentrations of the

at saturating menadione concentration. In the presence of saturating NADPH, the flavin reduction proceeds with a rate constant of 160 s^{-1} , which is at least 29-fold slower than estimates of the lower limit for the diffusion-limited rate constant characterizing NADPH binding to the enzyme under physiological conditions. Albery & Knowles have defined criteria for perfection in enzyme catalysis [Albery, W. J., & Knowles, J. R. (1976) *Biochemistry* 15, 5631–5640]. In the evolution of methylenetetrahydrofolate reductase, some catalytic power appears to have been sacrificed in the interest of metabolic control, since reduction of the enzyme-bound flavin by physiological concentrations of NADPH contributes to rate limitation in turnover with the physiological electron acceptor methylenetetrahydrofolate. However, the evolutionary pressure on the reductive half-reaction is minimized because this half-reaction is characterized by a pseudo-first-order rate constant (160 s^{-1}) that is approximately 3 times greater than the rate constant for reoxidation of the enzyme by saturating concentrations of methylenetetrahydrofolate (50 s^{-1}).

less thermodynamically stable substrate to the active center of the enzyme should be rate limiting in catalysis by a perfectly efficient enzyme, i.e., should be the most difficult step in the terminology of Ray (1983). Further, they suggested that evolution would act to maximize catalytic efficiency in enzymes, provided that they were not enzymes involved in metabolic control. However, they specifically considered that the imperatives of catalytic efficiency might be sacrificed to the imperatives of metabolic control and that enzymes involved in control might not show optimal catalytic efficiency. Since methylenetetrahydrofolate reductase is an allosterically regulated enzyme acting at a branch point of metabolism, it seemed of interest to assess its catalytic efficiency and, particularly, the catalytic efficiency of the irreversible segment of the overall reaction. To our knowledge, this is the first paper assessing the catalytic efficiency of an allosterically regulated enzyme involved in metabolic control.

Experimental Procedures

NADP⁺, NADPH, DL-*threo*-isocitrate, α -ketoglutarate, sodium borohydride, sodium borodeuteride (98% ²H), isocitrate

[†] From the Biophysics Research Division and the Department of Biological Chemistry, The University of Michigan, Ann Arbor, Michigan 48109. Received February 1, 1984. This work was supported in part by Research Grant GM 24908 awarded by the National Institute of General Medical Sciences.

[‡] Present address: Department of Biological Chemistry, University of Milan, 26 via Celoria, 20100 Milan, Italy.

¹ Abbreviations: $\text{CH}_2\text{-H}_4\text{folate}$, 5,10-methylenetetrahydrofolate; $\text{CH}_3\text{-H}_4\text{folate}$, 5-methyltetrahydrofolate; H_4folate , tetrahydrofolate; AdoMet, *S*-adenosylmethionine; ODS, octadecylsilane; EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography; ^D V , primary deuterium kinetic isotope effect on V_{max} ; ^D (V/K) , primary deuterium kinetic isotope effect on V_{max}/K_m ; ^T (V/K) , primary tritium kinetic isotope effect on V_{max}/K_m ; C_f , forward commitment to catalysis; C_r , reverse commitment to catalysis; R_f , the ratio of catalysis; k_{cat} , $V_{\text{max}}/[\text{total enzyme}]$; k_{obsd} , the observed pseudo-first-order rate constant; ^D K_{eq} , the primary deuterium kinetic isotope effect on the equilibrium constant; E_{ox} , oxidized methylenetetrahydrofolate reductase; E_{red} , two-electron-reduced methylenetetrahydrofolate reductase; $[E_T]$, total concentration of enzyme.

dehydrogenase from pig heart, and glutamate dehydrogenase from beef liver were purchased from Sigma. Sodium [³H]-borohydride (260 Ci/mol) and tritiated water (5 Ci/mL) were obtained from Amersham. Methylene tetrahydrofolate reductase from pig liver was prepared as previously described (Vanoni et al., 1983a; Daubner & Matthews, 1982a). NADPH-menadione and NADPH-CH₂-H₄folate oxidoreductase assays were performed as described earlier (Daubner & Matthews, 1982a; Matthews & Haywood, 1979), except that all assays were performed at pH 7.2.

Preparation of [4(R)-²H]NADPH and [4(R)-³H]NADPH. DL-[2-²H]isocitrate and DL-(2-³H)isocitrate were prepared by reduction of triethyl oxalosuccinate with sodium [²H]borohydride or sodium [³H]borohydride followed by base-catalyzed hydrolysis and purification on Dowex 1, chloride form, as described by Lowenstein (1963). [4(R)-²H]NADPH or [4(R)-³H]NADPH was prepared from the appropriate isotopically substituted isocitrate with the R-specific enzyme isocitrate dehydrogenase, by a modification of the procedure described by Rubenstein & Strominger (1974). NADP⁺, 60 μmol, and D-threo-isocitrate, 90 μmol (180 μmol of DL-threo-isocitrate), were dissolved in 10 mL of glycylglycine buffer, pH 7.5, containing 1 mM MgCl₂. Isocitrate dehydrogenase, 2 units, was added and the reaction mixture incubated at 25 °C until the 260 nm/340 nm absorbance ratio reached a minimum. Isocitrate dehydrogenase was removed by ultrafiltration through an Amicon PM-30 membrane.

Preparation of [4(S)-²H]NADPH and [4(S)-³H]NADPH. [4(R)-²H]NADPH or [4(R)-³H]NADPH was oxidized to [4-²H]NADP⁺ or [4-³H]NADP⁺ with glutamate dehydrogenase, a pro-S-specific dehydrogenase, as described by Hermes et al. (1982). The reaction was monitored at 340 nm, and when a plateau was reached, the glutamate dehydrogenase was removed by ultrafiltration through a PM-30 membrane. The [4-²H]NADP⁺ was purified on a 1.5 × 8 cm column of DEAE-52, previously equilibrated with 10 mM ammonium acetate, pH 6.5. The NADP⁺ was eluted with a 100-mL gradient of 0.01–0.50 M ammonium acetate, pH 6.5. Fractions were analyzed by conversion to NADPH with isocitrate dehydrogenase and isocitrate, and only fractions with 260 nm/340 nm absorbance ratios less than 2.4 were pooled. The pooled fractions were lyophilized to remove ammonium acetate. A portion of the [4-²H]NADP⁺ was subjected to proton magnetic resonance spectroscopy in a Bruker WM 360 spectrometer in order to determine the residual ¹H content at the 4-position of NADP⁺. The preparation used for these experiments contained 2.4% residual hydrogen at the 4-position. The isotopically substituted NADP⁺ was then reduced by isocitrate dehydrogenase in the presence of a 2-fold excess of unlabeled D-threo-isocitrate. At the completion of the reaction, isocitrate dehydrogenase was removed by ultrafiltration, and the [4(S)-²H]NADPH was used for kinetic studies without further purification. [4(S)-³H]NADPH was purified by chromatography on a column of DEAE-52, 1 × 12 cm, previously equilibrated with 10 mM glycylglycine buffer, pH 7.5. Elution of NADPH was effected with a 250-mL linear gradient of 0–4% NaCl in the same buffer. NADPH-containing fractions with 260 nm/340 nm absorbance ratios of less than 2.4 were pooled and, if necessary, were concentrated and desalted by ultrafiltration through an Amicon YC05 membrane.

Determination of the Stereospecificity of Oxidation of NADPH Catalyzed by Methylene tetrahydrofolate Reductase. A reaction mixture containing 125 μM [4(R)-³H]NADPH or [4(S)-³H]NADPH and 150 μM menadione in 50 mM

potassium phosphate buffer, pH 7.2, and 0.3 mM EDTA was incubated with methylene tetrahydrofolate reductase at 25 °C. The reaction was monitored at 343 nm. Aliquots, 1 mL, were withdrawn at the completion of the reaction, and the radioactivity released to the solvent was separated from radioactive NADPH and/or NADP⁺ by lyophilization and condensation of the solvent in a cold finger. A duplicate reaction mixture, lacking enzyme, was also incubated under the same conditions, and aliquots were removed and lyophilized to determine the extent of nonenzymatic release of tritium to the solvent. Aliquots obtained at the completion of both the enzymatic and control incubations were also applied to an Ultrasphere ODS HPLC column equilibrated with 0.1 M ammonium acetate buffer, pH 7.0. The column was eluted isocratically, and 1-mL fractions of the eluate were collected and analyzed by liquid scintillation counting.

Determination of the Primary Tritium Kinetic Isotope Effect on V/K. A reaction mixture containing 125 μM [4(S)-³H]NADPH (50 000–200 000 dpm/nmol) and 150 μM menadione in 50 mM potassium phosphate buffer, pH 7.2, containing 0.3 mM EDTA, was prepared and equilibrated at 25 °C. Enzyme was added to initiate the reaction, and a 1-mL aliquot was transferred to a cuvette and the reaction monitored in a spectrophotometer at 343 nm. At intervals, 1-mL aliquots of the reaction mixture were withdrawn, transferred to pre-chilled 5-mL round-bottom flasks, and rapidly frozen in a dry ice-acetone bath. These fractions were lyophilized, and the solvent was collected by condensation in a cold finger. The lyophilized residue was redissolved in 1 mL of glass-distilled water, and aliquots of both solvent and residue were analyzed in a liquid scintillation counter. A control incubation from which enzyme had been omitted was used to determine the extent of nonenzymatic release of tritium from [4(S)-³H]NADPH, and incubations in the presence of high levels of enzyme (0.175 units/mL) were used to determine the extent of tritium release associated with completion of the reaction. Determinations of the tritium content of solvent and residue were corrected for background dpm, for nonenzymatic release of tritium, and for the extent of tritium release at the completion of the reaction. The experimental data were analyzed by using eq 1, which has been derived from a similar equation

$$\tau(V/K) = \frac{\ln(1 - F_1)}{\ln(1 - F_2)} = k_H/k_T \quad (1)$$

presented in Melander & Saunders (1980). F_1 is defined as the fractional conversion of [4(S)-¹H]NADPH, determined from the absorbance at 343 nm at the time of removal of an aliquot from the reaction mixture according to eq 2. F_2 is

$$F_1(t) = \Delta A_{343t} / \Delta A_{343\infty} \quad (2)$$

defined as the fractional conversion of [4(S)-³H]NADPH, determined by measurement of the release of tritium to solvent according to eq 3, and k_H/k_T is the ratio of rate constants

$$F_2(t) = \frac{(\text{dpm in solvent})_t - (\text{dpm in blank})_t}{(\text{dpm in solvent})_\infty - (\text{dpm in blank})_\infty} \quad (3)$$

associated with the consumption of [4(S)-¹H]NADPH and [4(S)-³H]NADPH.

Determination of Primary and Secondary Deuterium Kinetic Isotope Effects on V and V/K. Steady-state analyses of NADPH-menadione oxidoreductase activity were performed in 50 mM potassium phosphate buffer, pH 7.2, containing 0.3 mM EDTA at 25 °C. The menadione concentration was 100 μM and the NADPH concentration was varied between 5 and 100 μM. A control experiment showed that

Table I: Stereospecificity of the Oxidation of NADPH Catalyzed by Methylene-tetrahydrofolate Reductase^a

substrate	dpm released to solvent		dpm retained in residue	
	+enzyme	-enzyme	+enzyme	-enzyme
[4(R)- ³ H]NADPH	32 000 (3%)	43 000 (4%)	991 000 (96%)	1 004 000 (96%)
	123 000 (4%)	101 000 (3%)	2 760 000 (97%)	2 750 000 (97%)
[4(S)- ³ H]NADPH	1 270 000 (89%)	78 000 (6%)	154 000 (11%)	1 180 000 (94%)
	7 280 000 (93%)	48 000 (1%)	565 000 (7%)	8 200 000 (99%)
	6 700 000 (92%)	71 000 (1%)	583 000 (8%)	7 250 000 (99%)

^aReaction conditions are described under Experimental Procedures.

the kinetics of oxidation of [4(S)-¹H]NADPH prepared in the same manner as deuterated substrate and of [4(S)-¹H]NADPH purchased from Sigma were indistinguishable. Oxidation of [4(S)-²H]NADPH and of [4(S)-¹H]NADPH purchased from Sigma were then compared by using the same enzyme preparation. Statistical analysis of steady-state data was accomplished with Cleland's HYPER program (Cleland, 1979), translated into PASCAL for use on an Ahmdahl 5860 computer. This program makes a least-squares fit to eq 4 by

$$v = \frac{V_{\max}}{1 + K_m/[S]} \quad (4)$$

assuming the velocities have equal variance and provides estimates of the standard errors associated with each determination of V and K_m .

Measurements of the α -secondary deuterium kinetic isotope effect associated with oxidation of [4(R)-²H]NADPH were performed under conditions identical with those utilized for measurements of the primary deuterium kinetic isotope effect. The data were initially analyzed by using Cleland's HYPER program, and when estimates of the α -secondary deuterium kinetic isotope effect on V and V/K were found to be identical, within the limits of precision of the data, the data were subjected to analysis with a program developed by Cleland that calculates a least-squares fit to eq 4 for both deuterated and protonated substrates, assuming the same values for K_m for both deuterated and protonated substrates.

Measurement of the Extent of Incorporation of Solvent Tritium into Residual NADPH during the NADPH–Menadione Oxidoreductase Reaction. Duplicate reaction mixtures, 2.0 mL, containing 125 μ M NADPH and 150 μ M menadione in 50 mM potassium phosphate buffer, pH 7.2, and 0.3 mM EDTA were prepared. Tritiated water, 0.1 mL, was added to one incubation mixture and H₂O, 0.1 mL, to the control. The reaction was initiated by addition of enzyme to each incubation mixture. A 25- μ L aliquot of the reaction mixture containing tritiated water was diluted into 100 mL of glass-distilled water, and duplicate 25- μ L aliquots were counted to determine the specific radioactivity of the solvent, which was 6450 dpm/nmol of hydrogen ion. When the reaction was 50% complete, as monitored at 343 nm in the control incubation, 500 nmol of NADPH was added to each incubation, and monitoring of the control was continued. The reaction mixture containing tritiated water was loaded onto a 1 \times 7 cm column of DEAE-52 previously equilibrated with 10 mM glycylglycine buffer, pH 7.5. The column was rinsed with 300 mL of the equilibrating buffer and then eluted with a 50-mL linear gradient of 0–4% NaCl in the same buffer. Fractions containing NADPH were pooled, diluted 10-fold, and rechromatographed on a 1 \times 14 cm column of DEAE-52 in glycylglycine buffer. Elution was effected with a 100-mL linear gradient of 0–4% NaCl in glycylglycine buffer. Fractions, 0.85 mL, were collected and analyzed for NADPH content spectrophotometrically and for tritium content by scintillation counting of 100- μ L aliquots.

Kinetic Simulations of Reductive Half-Reaction. Kinetic simulations were carried out on a Nova minicomputer as described by Matthews et al. (1977). Concentrations of reactants, intermediates, and products were calculated as a function of time. These concentrations were computed by numerical solutions of the set of differential equations describing eq 5 with user input of the values for initial reactant concentrations, molar absorbance coefficients for the reactants, intermediates, and products, and rate constants. The program is based on a second-order Runge–Kutta method (Carnahan et al., 1969).

Results

Stereospecificity of Oxidation of NADPH Catalyzed by Methylene-tetrahydrofolate Reductase. Table I summarizes the results of five experiments designed to determine the stereospecificity of NADPH oxidation in the NADPH–menadione oxidoreductase reaction catalyzed by methylene-tetrahydrofolate reductase. The first two experiments shown utilized [4(R)-²H]NADPH. It can be seen that the percentage of tritium released to solvent was the same in the presence and in the absence of enzyme, under conditions where NADPH oxidation in the presence of enzyme had proceeded to completion as judged by the absorbance changes at 343 nm. HPLC analysis of the solution containing product at the end of the reaction indicated that almost all the tritium remained associated with NADP⁺. The second set of the three experiments showed that oxidation of [4(S)-³H]NADPH resulted in the release of approximately 90% of the total tritium to the solvent. HPLC analysis of the product mixture showed that approximately 9% of the tritium eluted at the position of NADP⁺. On the basis of these experiments, we conclude that methylene-tetrahydrofolate reductase catalyzes the stereospecific removal of the *pro-S* hydrogen at the 4-position of NADPH and that subsequent exchange of this hydrogen with solvent occurs, either on the enzyme-bound flavin or after reduction of menadione to menadiol.

Determination of ^T(V/K) for NADPH–Menadione Oxidoreductase Reaction. Table II shows data from three experiments performed to determine the value for ^T(V/K) associated with the NADPH–menadione oxidoreductase reaction in the presence of saturating menadione. The mean value for all three experiments was 10.8 \pm 0.4 (standard error).

Determination of ^D(V/K) and ^DV Associated with Oxidation of [4(S)-²H]NADPH. Figure 1 represents a scatter graph of the data used to calculate ^D(V/K) and ^DV associated with the NADPH–menadione oxidoreductase reaction catalyzed by methylene-tetrahydrofolate reductase. The noncompetitive method of determination was used, and kinetic data were obtained with [4(S)-²H]NADPH and with [4(S)-¹H]NADPH obtained from Sigma and used without further purification. Control experiments established that no statistically significant effects on either V or K_m were seen when [4(S)-¹H]NADPH prepared in the same manner as [4(S)-²H]NADPH was compared with [4(S)-¹H]NADPH purchased from Sigma. The scatter graph shown in Figure 1 represents results obtained

Table II: Determination of $\tau(V/K)$ Associated with NADPH Oxidation^a

expt	electron acceptor	% conversion	$\tau(V/K)$
1	menadione	16.7	14.3
		21.5	9.78
		27.1	9.33
		30.2	7.63
		34.5	10.4
		38.0	12.6
		42.2	10.7
		52.0	9.90
2	menadione		10.6 ± 0.7^b
		14.7	10.7
		17.6	12.4
		19.6	7.81
		29.2	10.8
		39.3	7.91
		48.6	9.84
			9.9 ± 0.7^b
3	menadione	10.6	13.0
		16.0	12.7
		20.0	11.3
		25.0	10.5
		29.0	10.8
		33.0	11.0
		42.0	11.8
		45.0	11.4
			11.6 ± 0.3^b
			10.8 ± 0.4^c

^aReaction conditions are described under Experimental Procedures. The summary data are given as mean value \pm standard error of the mean. ^bMean values. ^cCombined mean values.

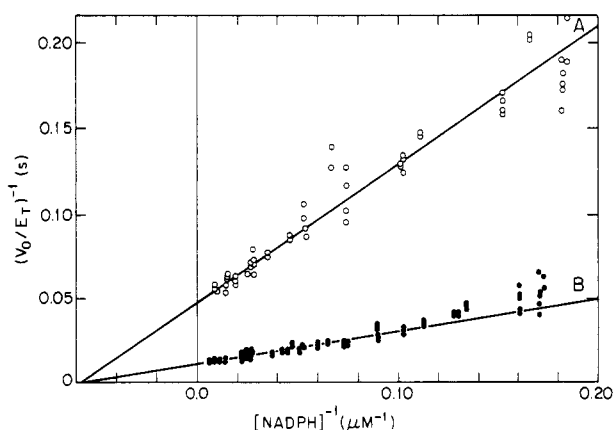


FIGURE 1: Scatter graph of the data points used to calculate $^D V$ and $^D(V/K)$: (A) data obtained with $[4(S)^{-2}H]NADPH$; (B) data obtained with $[4(S)^{-1}H]NADPH$. All data points correspond to initial velocity measurements at the indicated concentrations of NADPH and 100 μM menadione in 50 mM phosphate buffer, pH 7.2, containing 0.3 mM EDTA at 25 °C. The enzyme concentration used to measure the velocities with $[4(S)^{-1}H]NADPH$ was 0.81 nM, and that used to measure velocities with $[4(S)^{-2}H]NADPH$ was 3.22 nM. Control experiments established that the initial velocity was a linear function of enzyme concentration under these conditions. The solid lines represent least-squares fits to the data obtained by the HYPER program (see the text).

from five separate determinations of V and K_m with $[4(S)^{-1}H]NADPH$ and from three separate determinations with $[4(S)^{-2}H]NADPH$. Summaries of the results obtained from these individual determinations are given in Table III. As indicated by the scatter graph, the data from these individual determinations were sufficiently consistent that the best estimates of $^D V$ and $^D(V/K)$ were obtained by statistical analysis of all the data points. After analysis of the data with Cleland's HYPER program (Cleland, 1979), the data obtained with $[4(S)^{-2}H]NADPH$ were corrected for 2.4% residual 1H present at the 4-position of $[4(S)^{-2}H]NADPH$. The use of a least-

Table III: Measurements of $^D V$ and $^D(V/K)$ for NADPH-Menadione Oxidoreductase Reaction^a

substrate	V/E_T (s^{-1})	K_m^{-1} (NADPH) (μM)	$^D V$	$^D(V/K)$
$[4(S)^{-1}H]-NADPH$	90 ± 4	17.6 ± 2.7		
	101 ± 5	21.7 ± 2.6		
	94 ± 1	17.2 ± 0.6		
	113 ± 8	22.7 ± 2.9		
	96 ± 2	24.0 ± 1.1		
	94 ± 2^b	18.3 ± 0.8^b		
$[4(S)^{-2}H]-NADPH$	22.6 ± 1.0	22.4 ± 2.5		
	23.1 ± 1.0	19.5 ± 1.1		
	20.5 ± 0.4	15.0 ± 0.8		
	21.4 ± 0.4^b	17.4 ± 0.8^b	4.39 ± 0.14^b	4.16 ± 0.54^b
	19.7 ± 0.4^c		4.78 ± 0.15^c	4.54 ± 0.59^c

^aReaction conditions are described under Experimental Procedures. Values are expressed as mean values \pm standard errors of the mean. Corrected values have been corrected for 2.4% residual protium in $[4(S)^{-2}H]NADPH$. ^bMean values. ^cCorrected mean values.

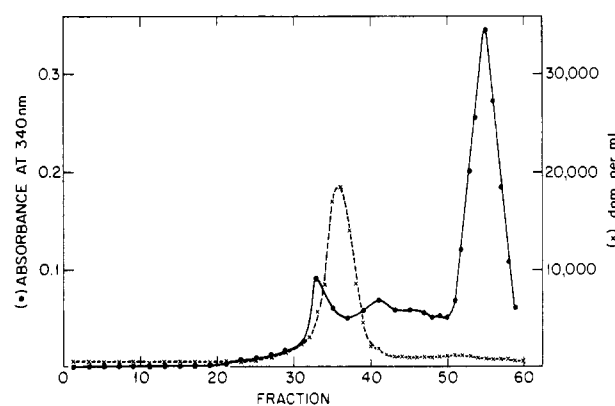


FIGURE 2: Elution profile of residual NADPH after catalysis of the NADPH-menadione oxidoreductase reaction in tritiated water (6450 dpm/nmol of H^+). Fractions were obtained during gradient elution from a DEAE-52 column (see the text) and were analyzed for absorbance and radioactivity (0.1-mL aliquots were counted). The peak centered at fraction 55 corresponded to authentic NADPH and could be oxidized by methylenetetrahydrofolate reductase in the presence of menadione. The radioactive peak centered at fraction 36 had absorbance maxima at 260 and 330 nm and was not a substrate for methylenetetrahydrofolate reductase in the presence of menadione.

squares analysis that assumes kinetic isotope effects of equal magnitude on V and V/K gave a similar value for the kinetic isotope effect and did not result in significant reduction in the standard errors associated with the data.

Measurement of Extent of Incorporation of Solvent Tritium into Residual NADPH during the NADPH-Menadione Oxidoreductase Reaction. When approximately 50% of the initial NADPH present had been oxidized in tritiated water (125 nmol out of 250), an additional 500 nmol of NADPH was added to the reaction mixture and the residual substrate was isolated. The results of the second chromatographic separation on DEAE-52 are shown in Figure 2. A radioactive impurity with absorbance maxima at 330 and 260 nm (fractions 31-40) preceded NADPH (fractions 51-59) in eluting from the column. We could not detect any radioactivity associated with NADPH. The identity of the latter compound was confirmed by enzymatic oxidation in the presence of methylenetetrahydrofolate reductase and menadione. The radioactive impurity did not show absorbance changes when incubated with enzyme in the presence of menadione, nor was tritium released into the solvent. We calculate that we could have easily detected the incorporation of 40 dpm/nmol of NADPH eluted from the column, which would have corresponded to 200

Table IV: Values for Kinetic Parameters in Scheme I^a

parameter	estimated values	method of determination
k_2'	160 s ⁻¹	rapid reaction studies of the reductive half-reaction
$K_d(\text{app})_{\text{NADPH}}$	28 μM	rapid reaction studies of the reductive half-reaction
V_{max}/E_0	164 s ^{-1b}	steady-state kinetics of NADPH–menadione oxidoreductase reaction
K_{mNADPH}	28 μM	steady-state kinetics of NADPH–menadione oxidoreductase reaction
k_4	1.7 × 10 ⁷ M ⁻¹ s ⁻¹	rapid reaction studies of the oxidative half-reaction

^a From Vanoni et al. (1983a). ^b Corrected for approximately 25% inactive enzyme as discussed in Vanoni et al. (1983a).

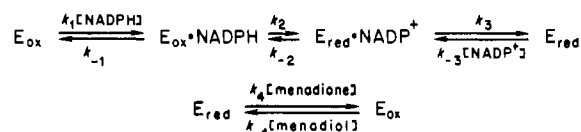
dpm/nmol of NADPH in the initial incubation mixture. Thus, incorporation of tritium into 3% of the residual NADPH would have been readily detectable.

Measurement of Secondary Kinetic Isotope Effect on V Associated with Oxidation of [4(R)-²H]NADPH in the NADPH–Menadione Oxidoreductase Reaction. When [4(R)-²H]NADPH oxidation and [4(R)-¹H]NADPH oxidation were compared in the NADPH–menadione oxidoreductase reaction, a value of 1.04 ± 0.01 was obtained for the secondary kinetic isotope effect on V and V/K , assuming the same K_{m} (16.9 ± 0.5 μM) for deuterated and protonated substrates. The assumption that K_{m} is the same is based on the fact that both primary and secondary kinetic isotope effects associated with oxidation of [4(S)-²H]NADPH arise from the same transition state, and the K_{m} values for oxidation of [4(S)-¹H]NADPH and [4(S)-²H]NADPH have been found to be the same within experimental error.

Discussion

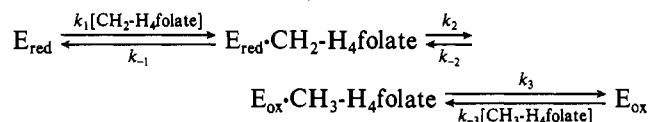
In the analyses that follow, we will utilize the kinetic scheme presented in Scheme I. Table IV presents measured values for several of the kinetic parameters listed in Scheme I.

An enzyme exhibiting catalytic perfection in the sense defined by Albery & Knowles (1976) catalyzes a reaction in which steps involved in covalent catalysis are not rate limiting (difficult) in the presence of physiological concentrations of substrates and products. Rather, for a reversible reaction, the approach of physiological concentrations of the less thermodynamically stable substrate to the enzyme active center is the most difficult step in catalysis. Thus, if methylenetetrahydrofolate reductase had been optimized for the catalytic efficiency of its reductive half-reaction, we would expect that the overall rate of this half-reaction would be limited by the rate of approach of NADPH to the oxidized enzyme at physiological concentrations of NADPH. All energy barriers associated with transition states connecting catalytic intermediates in the reaction should be lower than the barrier associated with substrate binding, and all free energies of such catalytic intermediates should be raised above the level of the product. In calculating the free energy profile for an optimally efficient reductive half-reaction, it is necessary to have estimates for the diffusion-limited rate constant for approach of NADPH to the enzyme (k_1), for the physiological concentration of NADPH in the cytoplasm of liver cells, and for ΔG° for the reductive half-reaction, where the standard state of NADPH and NADP⁺ is chosen to reflect the physiological levels of these metabolites. Since all our experiments have been performed at pH 7.2, we will calculate the standard free energies at that pH. Freeze-clamp measurements of NADPH and NADP⁺ concentrations in mouse liver give values of 200

Scheme I: A Minimal Kinetic Mechanism for NADPH–Menadione Oxidoreductase Activity^a

^a Under initial rate conditions, the following net rate constants can be calculated (Cleland, 1974): $k_4' = k_4[\text{menadione}]$; $k_3' = k_3$; $k_2' = k_2k_3/(k_{-2} + k_3)$; $k_1' = k_1k_2'[\text{NADPH}]/(k_{-1} + k_2')$ = $k_1k_2k_3[\text{NADPH}]/(k_2k_3 + k_{-2}k_2 + k_{-1}k_3)$. [Note that reoxidation of E_{red} is first order in menadione concentration over the concentration range employed for these studies (0–150 μM).] In terms of the net rate constants, the steady-state kinetic parameters are $V_{\text{max}}/E_T = 1/(1/k_2' + 1/k_3') = k_2k_3/(k_{-2} + k_2 + k_3)$, $V_{\text{max}}/K_M = k_1k_2k_3[E_0]/(k_2k_3 + k_{-1}k_{-2} + k_{-1}k_3)$, and $K_{\text{m}}(\text{NADPH}) = (k_{-1}k_{-2} + k_{-1}k_3 + k_2k_3)/[k_1(k_{-2} + k_2 + k_3)]$. This is a *minimal* kinetic scheme in that it does not include pre- or postcatalytic isomerization steps in the reductive half-reaction. While many dehydrogenases do show such steps, we have failed to obtain any evidence for such intermediates in stopped-flow studies of the reductive half-reaction, and the magnitudes of our observed primary kinetic isotope effects on V and V/K argue against kinetically significant pre- or postcatalytic conformational changes. For experiments with isotopically substituted NADPH, the following equations will govern the observed kinetic isotope effects (Northrop, 1977): $D(V/K) = (Dk_2 + C_f + C_r D K_{\text{eq}})/(1 + C_f + C_r)$, where $C_f = k_2/k_{-1}$ and $C_r = k_{-2}/k_3$; $DV = (Dk_2 + R_f + C_r D K_{\text{eq}})/(1 + R_f + C_r)$, where $R_f = k_2/k_3 + k_2/k_4$; $T(V/K) = (T k_2 + C_f + C_r T K_{\text{eq}})/(1 + C_f + C_r)$. Rearranging the formula for $K_{\text{m}}(\text{NADPH})$ in terms of R_f , C_f , and C_r , we obtain $K_{\text{m}}(\text{NADPH}) = (k_{-1}/k_1)/[(1 + C_f + C_r)/(1 + R_f + C_r)] = (k_{-1}/k_1)/[(D V - 1)/(D V/K - 1)]$ (Klinman & Matthews, 1984).

μM and 50 μM, respectively (Conway et al., 1983), and so we have chosen 200 μM as the standard-state concentration for both NADPH and NADP⁺ (Ray, 1983). A reasonable value for the ΔG° associated with the reductive half-reaction can be calculated from the midpoint potential of the $E_{\text{ox}}/E_{\text{red}}$ couple at pH 7.2. This value in turn can be derived from the Haldane relationship describing the reversible oxidative half-reaction with the physiological substrate CH₂-H₄folate and from the midpoint potential of the CH₂-folate/CH₃-H₄folate couple, which is -0.133 V at pH 7.2, calculated from Katzen & Buchanan (1965):



The K_d for the binding of CH₂-H₄folate to E_{red} is 88 μM, that for the binding of CH₃-H₄folate to E_{ox} is 20 μM, k_2 is 50 s⁻¹, and k_{-2} is 5.3 s⁻¹ (Vanoni et al., 1983a). From these values, we calculate a value for the midpoint potential of the $E_{\text{ox}}/E_{\text{red}}$ couple of -0.143 V at pH 7.2. This estimate is in good agreement with the value of -0.144 V calculated from a titration of enzyme with CH₃-H₄folate (Daubner & Matthews, 1982b). The calculated value, ΔG° , for the reductive half-reaction is then -9.0 kcal/mol. Finally, we estimate a minimum value for k_1 of 2.3 × 10⁷ M⁻¹ s⁻¹ (vide infra). This value is within the range of observed values for the association of enzymes with their physiological substrates, which is 10⁷–10⁸ M⁻¹ s⁻¹ (Hammes & Schimmel, 1970). At the physiological NADPH concentration, 200 μM, the pseudo-first-order rate constant for formation of the $E_{\text{ox}}\cdot\text{NADPH}$ complex would be 4600 s⁻¹. A number of other flavoproteins are reduced by saturating NADPH and NADH concentrations with apparent first-order rate constants of 3300 s⁻¹ or more (Massey et al., 1970; Matthews et al., 1979) and must have k_1 values in excess of 10⁷ M⁻¹ s⁻¹. The free-energy profile for optimally efficient catalysis of the reductive half-reaction of oxidized methyl-

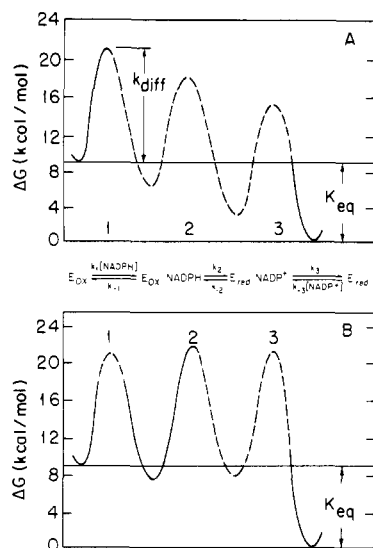


FIGURE 3: Notional free-energy profiles for a "perfect" enzyme (A) and for methylenetetrahydrofolate reductase (B). For purposes of comparison, we have assumed that both catalytic reactions involve the same value for $k_1[NADPH]$ (4600 s^{-1}) and the same $\Delta G^{\circ'}$ for the half-reaction (-9.0 kcal/mol). Solid lines have been used to define regions of the profile that are constrained [by the requirements for optimal catalytic efficiency (A) or by the experimental data (B)], and dashed lines have been used to define regions of the profile that are not as constrained. In (B), the dashed barrier heights are maximal and may actually be significantly lower than those shown.

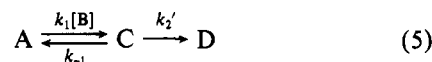
enetetrahydrofolate reductase with NADPH is shown in Figure 3A.² Since barrier 1 is higher than barrier 2 for a "perfect" enzyme at physiological NADPH concentration, the $E_{ox} \cdot NADPH$ complex, once formed, should preferably undergo oxidoreduction to form the $E_{red} \cdot NADP^+$ complex rather than dissociate to form E_{ox} and NADPH. Thus, the "perfect" enzyme should show suppression of the V/K_{NADPH} isotope effect associated with the use of $[4(S)^{-2}H]NADPH$ or $[4-(S)^{-3}H]NADPH$ due to a high forward commitment to catalysis, C_f (Northrop, 1977).

We have previously shown that reoxidation of reduced enzyme by $120 \mu\text{M}$ menadione proceeds at a rate that is about 2100 s^{-1} , or 13-fold faster than the rate of reduction of oxidized enzyme by saturating NADPH (Vanoni et al., 1983a), and that the rate of reoxidation of reduced enzyme is first order in menadione concentration. Catalysis of the NADPH–menadione oxidoreductase reaction has also been shown to proceed by a Ping Pong Bi Bi mechanism. Thus, studies of steady-state kinetics of the NADPH–menadione oxidoreductase reaction can be used to probe events associated with the reductive half-reaction. As documented under Results, we see little or no suppression of the kinetic isotope effects on V or V/K . Furthermore, at $200 \mu\text{M}$ NADPH, which corresponds to the estimated cytoplasmic concentration of NADPH, the rate of reduction of the enzyme by NADPH and the rate of turnover in the NADPH–menadione oxidoreductase reaction are essentially zero order in NADPH. Thus, we conclude that methylenetetrahydrofolate reductase is *not* exhibiting

optimal catalytic efficiency in catalyzing the oxidation of NADPH.

To put these findings on a more quantitative basis, it is necessary to calculate an actual free-energy profile for the reductive half-reaction, in order to compare it with the profile indicated for optimal catalytic efficiency. We note first of all that the measured primary deuterium kinetic isotope effects on V and V/K are the same. Thus, any suppression of $^D V$ by the ratio of catalysis, R_f , must be balanced by corresponding suppression of $^D V/K$ by the forward commitment, C_f . (These terms are defined in Scheme I.) Under the conditions of the initial steady-state kinetic measurements used to determine these kinetic isotope effects, $C_f = k_2/k_{-1}$ and $R_f = k_2/k_3$ since menadione is present at saturating concentration and $NADP^+$ is initially absent. Thus, equality in the measured values of $^D V$ and $^D V/K$ implies that $C_f \approx R_f$, so that either $k_2 \ll k_{-1}$ and $k_2 \ll k_3$ or $k_{-1} = k_3$. In either case, the K_m measured for NADPH in steady-state turnover will be equal to the K_d for NADPH.

Our stopped-flow studies on the reductive half-reaction yield an apparent K_d for NADPH ($28 \mu\text{M}$) that is in good agreement with the K_m measured in steady-state turnover (Vanoni et al., 1983a). The meaning of the apparent K_d measured for an enzymatic half-reaction by rapid reaction kinetics has been discussed by Strickland et al. (1975). Our reductive half-reaction (shown in Scheme I) can be reduced to one of the systems discussed by Strickland and his co-workers if we assume that $k_{-3}[NADP^+] \ll k_3$.⁴ We can therefore represent the reductive half-reaction as shown in eq 5. Strickland and



colleagues used analogue-simulation studies to evaluate the meaning of the apparent K_d for this reaction in terms of the kinetic constants. They showed that if measurements were made at sufficiently high levels of $[B_0]$, for the case in which k_2' has approximately the same magnitude as k_{-1} , the measured $K_d(\text{app})$ is equal to $(k_{-1} + k_2')/k_1$.⁵ Of course, if k_{-1} is actually larger than 160 s^{-1} , $K_d(\text{app})$ will retain its significance and will approach a true K_d for values of $k_{-1} \gg k_2'$, and the agreement between K_m and $K_d(\text{app})$ indicates that this is the case. Since $k_{-1} \gg k_2'$ and $k_2' = 160 \text{ s}^{-1}$, we can assume a *minimum* value for k_{-1} of 480 s^{-1} and can calculate a *minimum* value for k_1 of $2.3 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$. As mentioned above,

³ $R_f = k_2/k_3' + k_2/k_4' \approx k_2/k_3$ (since $k_4' \gg k_2$).

⁴ This assumption is justified by the fact that the $NADP^+$ present during the reductive half-reaction never exceeds the initial enzyme concentration ($3.2 \mu\text{M}$ in these experiments) and by the observation that the presence of $670 \mu\text{M}$ $NADP^+$ does not lead to inhibition of steady-state turnover in the NADPH– $\text{CH}_2\text{-H}_4\text{folate}$ oxidoreductase reaction when NADPH is saturating (Daubner, 1982).

⁵ At lower concentrations of B_0 , double-reciprocal plots of k_{obsd} vs. $[B_0]$ are concave upward, and the $K_d(\text{app})$ is difficult to evaluate. The region of curvature corresponds to situations in which there is a significant lag phase associated with the formation of D. To ensure that our stopped-flow measurements would yield a $K_d(\text{app})$ equal to $(k_{-1} + k_2')/k_1$, we have simulated this reaction under conditions prevailing in the stopped-flow studies, assuming a *minimal* value of k_{-1} , viz., $k_{-1} = 1.14 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$, $k_{-1} = 160 \text{ s}^{-1}$, $k_2' = 160 \text{ s}^{-1}$, $[A_0] = 2.4 \mu\text{M}$ (active enzyme), and $[B_0] = 10.4, 16.1, 32.9, \text{ or } 161 \mu\text{M}$. The simulated traces showed only a small lag phase, which lay largely within the 3-ms dead time associated with actual stopped-flow measurements. Semilogarithmic plots of $d[A + C]/dt$ are apparently linear over five to six half-lives. Double-reciprocal plots of k_{obsd} vs. $[B_0]$ appear linear within the limited range of B_0 used in the stopped-flow studies and simulations, and the extrapolated value of $K_d(\text{app})$ is given by $(k_2' + k_{-1})/k_1$ as predicted by the solution to eq 5 obtained by making the steady-state assumption that $d[C]/dt = 0$ (Strickland et al., 1975). Thus, we feel that we are justified in using the measured value for $K_d(\text{app})$ to calculate k_1 .

² In their original analysis, Albery & Knowles (1976) also suggested that the equilibrium constant characterizing central complex interconversion should be close to unity. Chin (1983) has recently questioned whether that conclusion is valid for reactions that are effectively irreversible. In fact, for highly exergonic reactions or for reactions that proceed under effectively irreversible conditions *in vivo* due to product trapping, catalytic perfection may *require* a substantial free-energy decrease to be associated with central complex interconversion (R. Raines and J. R. Knowles, personal communication). Such a profile is indicated in Figure 3A.

this estimated value is within the range expected for a diffusion-limited rate constant for an encounter of a protein with its physiological substrate. At physiological NADPH concentration, the minimum value for $k_1[\text{NADPH}]$ is 4600 s^{-1} , corresponding to a free energy of activation of no more than 12.4 kcal/mol. The aforementioned requirement that $k_2/k_{-1} = k_2/k_3$ implies that $k_3 \geq 480 \text{ s}^{-1}$ and, hence, that $k_2 \ll k_3$. This is substantiated by the stopped-flow measurements of k_2' for the reductive half-reaction, which are in good agreement with k_{cat} measurements during steady-state turnover. Inspection of Scheme I indicates that k_2' must be less than k_3 for k_2' to be equal to k_{cat} . Thus, the barrier for NADPH dissociation from $\text{E}_{\text{ox}}\cdot\text{NADPH}$ (k_{-1}) must be of approximately equal height as the barrier for NADP^+ dissociation from $\text{E}_{\text{red}}\cdot\text{NADP}^+$, and both must be less than 13.8 kcal/mol. Given a K_d for NADPH of $28 \mu\text{M}$, we calculate that $\text{E}_{\text{ox}}\cdot\text{NADPH}$ must lie 1.3 kcal below E_{ox} on the free-energy profile in the presence of $200 \mu\text{M}$ NADPH (our standard state). The free-energy profile will be completely defined if the height of barrier 3 can be related to that of barrier 2. Since we observe primary deuterium kinetic isotope effects on V and V/K of approximately 5 and the maximum semiclassical value for the intrinsic deuterium kinetic isotope effect is about 10, we may assume that the intrinsic isotope effect is suppressed by no more than a factor of 2. Inspection of Scheme I indicates that the magnitudes of the observed primary deuterium kinetic isotope effects on V and V/K imply that $R_f + C_r = C_f + C_r \leq 1$. Thus, $k_2 \leq k_3$ and $k_{-2} \leq k_3$. In agreement with these conclusions, we are unable to detect incorporation of solvent tritium into residual NADPH. If barrier 3 were as high as barrier 2 and if $\text{E}_{\text{red}}\cdot\text{NADP}^+$ were capable of exchange with solvent (a reasonable assumption for a flavoprotein), then a substantial fraction of the $\text{E}_{\text{red}}\cdot\text{NADP}^+$ produced should exchange with solvent and then return to $\text{E}_{\text{ox}}\cdot\text{NADPH}$. Dissociation of the complex would then lead to detectable tritium incorporation into the residual substrate. However, observation of incorporation of solvent tritium into residual NADPH could also be prevented if $\text{E}_{\text{red}}\cdot\text{NADP}^+$ were to have the flavin shielded from solvent so that exchange did not occur until after dissociation of NADP^+ . Thus, this experiment is suggestive rather than conclusive. Finally, we observe only a small secondary kinetic isotope effect (1.04 ± 0.01) when $[4(R)\text{-}^2\text{H}]\text{NADPH}$ is used as substrate in the NADPH–menadione oxidoreductase reaction. In the absence of knowledge of the magnitude of the α -secondary equilibrium isotope effect associated with the oxidation of enzyme-bound $[4(R)\text{-}^2\text{H}]\text{NADPH}$, this experiment cannot be interpreted rigorously. However, the α -secondary equilibrium isotope effect associated with oxidoreduction of $[4\text{-}^2\text{H}]\text{NAD}^+$ by yeast alcohol dehydrogenase has been found to be 1.12 (Cook et al., 1980). In that case, the observed secondary kinetic isotope effect associated with NADH oxidation was in fact greater than 1.12, presumably due to coupled motion of the α -secondary hydrogen and the primary hydrogen in the transition state for hydride transfer (Cook et al., 1981). Thus, the relatively low α -secondary kinetic isotope effect we observe probably precludes substantial reversal of the oxidoreduction and also appears to be too small for a symmetrical transition state with coupled motion of the primary and α -secondary hydrogens. Thus, all our evidence is consistent with the conclusion that barrier 3 is lower than barrier 2 and that reduction of the enzyme-bound flavin by NADPH is an exergonic reaction with a relatively early transition state.

Finally, we note that the magnitudes of $^{\text{D}}(V/K)$ and $^{\text{T}}(V/K)$, 4.5 ± 0.6 and 10.8 ± 0.4 , have ranges that lie within the ratio

predicted by the Swain–Schaad relationship (Swain et al., 1958).⁶ This agreement does not *prove* that the full intrinsic isotope effect is being measured by these parameters, since the percentage deviation from the Swain–Schaad relationship due to suppression of the isotope effects on V/K is very small for observed isotope effects of this magnitude (Albery & Knowles, 1977), but our data are consistent with the presumption that the observed isotope effects are, in fact, the intrinsic ones.

Thus, the reductive half-reaction catalyzed by methylenetetrahydrofolate reductase is not optimized for catalytic efficiency. In particular, the overall rate of reduction in the presence of physiological concentrations of NADPH proceeds at least 29-fold more slowly than the estimated value for the first-order rate constant characterizing formation of the $\text{E}_{\text{ox}}\cdot\text{NADPH}$ complex under these conditions. We may ask why such suboptimal catalytic efficiency is tolerable, or even desirable, in this enzyme. We note first of all that the rate of reduction of the enzyme by saturating concentrations of NADPH (160 s^{-1}) is considerably faster than the rate of reoxidation of the enzyme by saturating concentrations of its physiological substrate $\text{CH}_2\text{-H}_4\text{folate}$ (50 s^{-1}). The effect of a 29-fold decrease in the observed rate of reduction vis à vis the theoretical maximum translates to a 1.3-fold decrease in the actual rate of turnover with the physiological substrate. This insulation of a half-reaction that is not rate limiting (sensitive) in overall turnover from the pressures of evolution may occur quite generally in enzymes that utilize Ping Pong Bi Bi kinetic mechanisms, whether or not they are allosterically regulated.

Second, we note that allosteric regulation is typically exerted on reactions that proceed under conditions that are effectively irreversible in vivo. In the present instance, the overall physiological reaction can be subdivided into an irreversible reductive half-reaction and a reversible oxidative half-reaction. We have shown that AdoMet, the allosteric inhibitor of this reaction, leads to a 50 000-fold decrease in the observed rate of reduction of the enzyme without any indication of an elevated K_m for NADPH (Vanoni et al., 1983b). It is not yet clear whether AdoMet exerts comparable effects on the rate constants for interaction of the reduced enzyme with either $\text{CH}_2\text{-H}_4\text{folate}$ or menadione. Thus, oxidoreduction of the $\text{E}_{\text{ox}}\cdot\text{NADPH}$ complex to form $\text{E}_{\text{red}}\cdot\text{NADP}^+$ represents one site for allosteric regulation of the activity of this enzyme, and the relative inefficiency of this catalytic step may reflect constraints related to the regulatory process.

Acknowledgments

We thank Dr. Harry Eckerson, Warner-Lambert Co., for translating Cleland's programs for kinetic analysis into PASCAL and Professor David P. Ballou, The University of Michigan, for his assistance with kinetic simulations of the reductive half-reaction. We also thank Professors Jeremy Knowles at Harvard University and W. W. Cleland at the University of Wisconsin for their helpful comments on the manuscript.

References

- Albery, W. J., & Knowles, J. R. (1976) *Biochemistry* 15, 5631–5640.
- Albery, W. J., & Knowles, J. R. (1977) *J. Am. Chem. Soc.*

⁶ The value for $^{\text{D}}K_{\text{eq}}$ associated with the transfer of deuterium from NADPH to H_2O is 0.98 (Cleland, 1980), and hence, its effect on $^{\text{D}}(V/K)$ can be neglected.

- 99, 637-638.
- Carnahan, B., Luther, H. A., & Wilkes, J. O. (1969) *Applied Numerical Methods*, Wiley, New York.
- Chin, J. (1983) *J. Am. Chem. Soc.* 105, 6502-6503.
- Cleland, W. W. (1979) *Methods Enzymol.* 63, 103-138.
- Cleland, W. W. (1980) *Methods Enzymol.* 64, 104-125.
- Conway, J. G., Kauffman, F. C., & Thurman, R. G. (1983) *J. Biol. Chem.* 258, 3825-3831.
- Cook, P. F., Blanchard, J. S., & Cleland, W. W. (1980) *Biochemistry* 19, 4853-4858.
- Cook, P. F., Oppenheimer, N. J., & Cleland, W. W. (1981) *Biochemistry* 20, 1817-1825.
- Daubner, S. C. (1982) Ph.D. Thesis, The University of Michigan.
- Daubner, S. C., & Matthews, R. G. (1982a) *J. Biol. Chem.* 257, 140-145.
- Daubner, S. C., & Matthews, R. G. (1982b) in *Flavins and Flavoproteins* (Massey, V., & Williams, C. H., Eds.) pp 165-192, Elsevier/North-Holland, New York.
- Hammes, G. B., & Schimmel, P. R. (1970) *Enzymes*, 3rd Ed. 2, 67-114.
- Hermes, J. D., Roeske, C. A., O'Leary, M. H., & Cleland, W. W. (1982) *Biochemistry* 21, 5106-5114.
- Katzen, H. M., & Buchanan, J. M. (1965) *J. Biol. Chem.* 240, 825-835.
- Klinman, J. P., & Matthews, R. G. (1984) *J. Am. Chem. Soc.* (in press).
- Kutzbach, C., & Stokstad, E. L. R. (1971) *Biochim. Biophys. Acta* 250, 459-477.
- Lowenstein, J. (1963) *Methods Enzymol.* 4, 875-876.
- Massey, V., Matthews, R. G., Foust, G. P., Howell, L. G., Williams, C. H., Jr., Zanetti, G., & Ronchi, S. (1979) in *Pyridine Nucleotide Dependent Dehydrogenases* (Sund, H., Ed.) pp 393-411, Springer-Verlag, Berlin.
- Matthews, R. G., & Haywood, B. J. (1979) *Biochemistry* 18, 4845-4851.
- Matthews, R. G., Ballou, D. P., Thorpe, C., & Williams, C. H., Jr. (1977) *J. Biol. Chem.* 252, 3199-3207.
- Matthews, R. G., Ballou, D. P., & Williams, C. H., Jr. (1979) *J. Biol. Chem.* 254, 4947-4981.
- Melander, L., & Saunders, W. H., Jr. (1980) *Reaction Rates of Isotopic Molecules*, Wiley-Interscience, New York.
- Northrop, D. B. (1977) in *Isotope Effects on Enzyme-Catalyzed Reactions* (Cleland, W. W., O'Leary, M. H., & Northrop, D. B., Eds.) pp 122-152, University Park Press, Baltimore.
- Palcic, M. M., & Klinman, J. P. (1983) *Biochemistry* 22, 5957-5966.
- Ray, W. J. (1983) *Biochemistry* 22, 4625-4637.
- Rubenstein, P. A., & Strominger, J. L. (1974) *J. Biol. Chem.* 249, 3782-3788.
- Strickland, S., Palmer, G., & Massey, V. (1975) *J. Biol. Chem.* 250, 4048-4052.
- Swain, C. G., Stivers, E. C., Revwer, J. F., Jr., & Schaad, L. J. (1958) *J. Am. Chem. Soc.* 80, 5885-5893.
- Vanoni, M. A., Ballou, D. P., & Matthews, R. G. (1983a) *J. Biol. Chem.* 258, 11510-11514.
- Vanoni, M. A., Daubner, S. C., Ballou, D. P., & Matthews, R. G. (1983b) in *The Chemistry and Biology of Pteridines* (Blair, J. A., Ed.) pp 235-239, de Gruyter, Berlin.

Photoreaction Center of *Ectothiorhodospira* sp. Pigment, Heme, Quinone, and Polypeptide Composition[†]

Suzie Lefebvre, Rafael Picorel, Yves Cloutier, and Gabriel Gingras*

ABSTRACT: The photoreaction center of *Ectothiorhodospira* sp., a member of the family Chromatiaceae, was isolated from its photosynthetic membranes with a yield of approximately 25-35%. The preparation is free of antenna bacteriochlorophyll and associated proteins. Its pigment complement is 4 mol of bacteriochlorophyll, 2 mol of bacteriopheophytin, and 1 mol of spirilloxanthin. On this basis, its molar extinction coefficient was calculated. Whereas chromatophores contain both ubiquinone and menaquinone, the photoreaction center contains only menaquinone, which, therefore, probably is the primary electron acceptor. The protein is composed of three different subunits of apparent molecular weights of 39 100, 31 300, and 24 800. The sum of these weights is very close

to the protein minimal molecular weight of the photoreaction center based on its molar extinction coefficient and amino acid content. This indicates a 1:1:1 molar stoichiometry. Four moles of heme *c* per mole of photoreaction center and cytochromes *c*-555 and *c*-552 are also present. Specific staining after polyacrylamide gel electrophoresis shows that all the heme is bound to the 39K polypeptide. A good amino acid composition homology is found between the L and M polypeptides from *Ectothiorhodospira* sp. and from *Rhodospirillum rubrum*. A membrane-bound cytochrome from *Chromatium vinosum* seems to be homologous to the heaviest subunit of the former two organisms.

Ectothiorhodospira is a bacteriochlorophyll *a* containing member of the family Chromatiaceae. These bacteria carry

[†]From the Département de Biochimie, Université de Montréal, Montréal, Québec, Canada H3C 3J7. Received January 24, 1984. Supported by a grant from the Natural Sciences and Engineering Research Council of Canada, by a bursary from the F.R.S.Q. (Québec) to S.L., and by a fellowship from the C.S.I.C. (Spain) to R.P.

out anoxygenic photosynthesis in which sulfide is oxidized to sulfate via a series of intermediates among which is elemental sulfur. Unlike the other Chromatiaceae, *Ectothiorhodospira* does not accumulate sulfur granules in its cytoplasm. Its photosynthetic machinery is localized in cytoplasmic membranes piled up as lamellar stacks (Truper & Pfennig, 1978; Remsen, 1978).