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Kinetic Studies of the Mechanism of Pyridine Nucleotide Dependent Reduction of Yeast Glutathione Reductase†

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ABSTRACT: The mechanism of electron transfer from reduced pyridine nucleotide to the active site of the flavoenzyme glutathione reductase has been studied by stopped-flow kinetic methods. Three spectrophotometrically and kinetically distinguishable steps are detected at 5 °C during the conversion of the oxidized form of the enzyme into the catalytically active two-electron reduced form. The very rapid first step is essentially complete within the dead time (~5 ms) of the stopped-flow apparatus. The absorption spectrum of the intermediate enzyme species produced in this initial step exhibits both a low-intensity band at long wavelength between 650 and 730 nm and a concomitant partial bleaching of the flavin absorbance peak at 462 nm. This transient intermediate is identified as a charge-transfer complex between reduced nicotinamide adenine dinucleotide phosphate (NADPH) and the oxidized flavin adenine dinucleotide (FAD) at the enzyme active site. The second kinetically detectable step of the reductive half-reaction has a limiting rate constant of 153 s^{-1} and shows saturation kinetics with K_d = 8.3 μM. While the first step is not detectably affected by the substitution of (4*S*)-[^{2}H]NADPH, the second step exhibits a kinetic isotope effect of 2.7, identifying the latter as the step in which the C–H bond of NADPH is broken. The third detectable step has a limiting rate constant of 68 s^{-1}; it is this step which leads to formation of the 530-nm absorbance band which is characteristic of the two-electron reduced form of the enzyme. The apparent rate constant for this step is decreased 1.8-fold when (4*S*)-[^{2}H]NADPH is used in place of NADPH. It is demonstrated that the apparent isotope effect of 1.8 on this step is a consequence of the preceding step having become rate limiting and not due to an intrinsic kinetic isotope effect on the third step. Steady-state kinetic studies at 25 °C show a kinetic isotope effect of 1.7 when [^{2}H]NADPH is substituted for NADPH, indicating that the reductive half-reaction, and specifically the step leading to the formation of the 530-nm band, is rate limiting to the overall catalytic reaction. 1,4,5,6-Tetrahydronicotinamide adenine dinucleotide phosphate is shown to be an inhibitor of glutathione reductase competitive with respect to NADPH, with K_i = 16 μM. Despite the high affinity of the enzyme for this enzymatically inactive pyridine nucleotide analogue, its binding to the enzyme does not significantly perturb the absorption spectrum of the enzyme-bound FAD. Apparently the binding of pyridine nucleotide alone is insufficient to effect the spectral perturbation produced within the dead time of the stopped-flow apparatus when NADPH binds.

The active site of the flavoenzyme glutathione reductase (NADPH:GSSG oxidoreductase, EC 1.6.4.2) contains both FAD and a redox-active protein disulfide bond; both are involved in the transfer of reducing equivalents from pyridine nucleotide to oxidized glutathione (Massey & Williams, 1965; Williams, 1976). Despite the potential for accepting a total of four electrons at the active site, the enzyme shuttles between an oxidized (E)[1] and a two-electron reduced (EH_2) form during catalysis (Massey & Williams, 1965; Bulger & Brandt, 1971a). The visible absorption spectrum of EH_2 is similar to that of the related flavoenzyme lipoamide dehydrogenase (Williams, 1976) and is thought to represent an internal charge-transfer complex between a thiolate anion, generated upon reduction of the protein disulfide bond, and the oxidized FAD (Thorpe & Williams, 1976; Massey & Ghisla, 1974; Kosower, 1966). With lipoamide dehydrogenase, Wilkinson & Williams (1979a,b) have demonstrated that EH_2 is a mixture of three species of which the corresponding charge-transfer complex is one; the other two are a form with the thiolate presumably protonated and hence unable to charge transfer with the oxidized FAD and a form with the flavin reduced and the disulfide oxidized. Anaerobic reduction of lipoamide dehydrogenase by dihydrolipoamide leads initially to the charge-transfer species, which subsequently relaxes to an equilibrium mixture of the three forms.

Recent X-ray crystallographic studies of erythrocyte glutathione reductase (Schulz et al., 1978) suggest that the binding site for the pyridine nucleotide and the redox-active protein disulfide bond lie on opposite sides of the isoalloxazine ring of the FAD, precluding direct electron transfer from NADPH to the protein disulfide. This supports the suggestion (Massey, 1963; Sanadi, 1963; Thorpe & Williams, 1976) that catalysis involves sequential electron transfer from reduced pyridine nucleotide to FAD to the protein disulfide to oxidized glutathione.

In an attempt to define the molecular events which occur during the catalysis of electron transfer by glutathione reductase and to obtain kinetic and spectroscopic evidence for the involvement of the FAD moiety in the transfer of electrons from reduced pyridine nucleotide to the protein disulfide, we have undertaken stopped-flow kinetic studies of the reductive half-reaction. NADPH is the preferred pyridine nucleotide substrate for glutathione reductase, but NADH can also act as an electron donor (Scott et al., 1963; Woodin & Segel, 1968; Bulger & Brandt, 1971a). Bulger & Brandt (1971a) showed that with NADH the rate-limiting step in the formation of EH_2 from E is the bimolecular combination of NADH with E to form a productive complex. Thus the NADH-dependent reduction of glutathione reductase is not suited for the study

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[1] Abbreviations used: E, EH_2, and EH_4, oxidized, two-electron reduced, and four-electron reduced forms, respectively, of glutathione reductase; AcPyADH, reduced 3-acetylpyridine adenine dinucleotide; PyAlADH, reduced 3-pyridinealdehyde adenine dinucleotide; DSS, sodium 4,4-dimethyl-4-silapentane-5-sulfonate; EDTA, ethylenediaminetetraacetic acid; BASF, Badische Anilin- und Sodafabrik.

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of electron transfer from pyridine nucleotide to flavin; steps subsequent to the formation of the NADH–enzyme complex are kinetically invisible. However, preliminary experiments (Bulger, 1971) using NADPH as substrate indicated that reduction by this pyridine nucleotide was kinetically more complex.

In this paper we report on the interactions of NADPH and two NADPH analogues with glutathione reductase. We are able to postulate that there is a minimum of three spectrophotometrically distinguishable steps in the reductive half-reaction to generate EH_2, the first of which involves formation of a preequilibrium charge-transfer complex between NADPH and the oxidized enzyme flavin. Experiments with (4*S*)-[^{2}H]NADPH identify which step corresponds to hydride transfer and suggest that the reductive half-reaction is rate limiting in the overall catalytic reaction. This contrasts with results obtained with lipoamide dehydrogenase (Matthews et al., 1977, 1979), where reoxidation of EH_2 by lipoamide is rate limiting in the NADH–lipoamide reductase reaction. A preliminary account of part of this work has been presented (Huber & Brandt, 1978).

Experimental Procedures

Materials

Yeast glutathione reductase (type III) was obtained from Sigma Chemical Co. If the specific activity of the enzyme was at least 250 IU/mg after dialysis against standard buffer (60 mM sodium phosphate, pH 7.6, containing 3 mM EDTA), it was used without further purification. Otherwise, the enzyme was purified further as previously described (Moroff & Brandt, 1973). Bovine liver glutamate dehydrogenase (3 times crystallized) was obtained from Sigma Chemical Co. and used directly. NADPH and $NADP^+$ were purchased from both Calbiochem and Sigma Chemical Co. [2-^{2}H]Glutamic acid (>98% labeled) was obtained from Aldrich Chemical Co. while deuterium oxide (>99.7% isotopically pure) was from Merck & Co.

Methods

Assays and Spectra. Standard assays of glutathione reductase activity were performed at pH 7.6 as described by Massey & Williams (1965). Anaerobic conditions for spectral work were achieved by either of two methods. In the first, Thunberg cuvettes were repeatedly flushed with nitrogen gas and then evacuated. The N_2 was scrubbed of contaminating oxygen by passage through a vanadate–sulfuric acid solution (Meites & Meites, 1948) and then through a water trap. Alternatively, 1-mL cuvettes fitted with gray butyl rubber septa and test tubes fitted with rubber serum caps were attached via syringe needles to an all-glass manifold for the cycles of flushing with N_2 and evacuation. In this case the N_2 was passed through an activated BASF catalyst (Ace Glass Inc.) in order to remove traces of oxygen. Gas-tight Hamilton syringes were used to transfer anaerobic solutions to anaerobic cuvettes. Spectra were recorded as described previously (Bulger & Brandt, 1971a).

Extinction coefficients employed for the spectrophotometric determination of reactant concentrations were 6.22 mM^{-1} cm^{-1} at 340 nm for NADPH and NADH (Horecker & Kornberg, 1948), 13.6 mM^{-1} cm^{-1} at 288 nm for tetrahydro-NADP (Dave et al., 1968), and 11.3 mM^{-1} cm^{-1} at 462 nm for enzyme-bound FAD (Massey & Williams, 1965).

Instrumentation. A Durrum-Gibson stopped-flow spectrophotometer was used for stopped-flow experiments. The photomultiplier was coupled to either a Tektronix RM 564 storage oscilloscope or an American Instruments Co. DASAR system. In the first instance the time-dependent change in transmittance of the reaction mixture was recorded on Polaroid photographs. Alternatively, the DASAR system was used to digitize the photomultiplier output signal, and the results were printed out on a teletype. For both systems, an apparent first-order rate constant, k_{obsd}, was evaluated from the data as described previously (Bulger & Brandt, 1971a).

NMR spectra were recorded on either a Perkin-Elmer R32 NMR spectrometer with a 90-MHz field strength (DSS used as the internal reference) or a Varian XL-100 spectrometer with a 100-MHz field strength (locked onto deuterium oxide). Samples were dissolved in D_2O and all NMR spectra were recorded at 35 °C.

Calculations of the predicted time-dependent variation of the concentrations of the components of eq 1 were performed by computer with Fortran programs run on the MNF compiler at the Purdue University Computing Center.

Preparation of (4S)-[^{2}H]NADPH. This analogue was synthesized by use of glutamate dehydrogenase, [2-^{2}H]glutamic acid, and $NADP^+$. Glutamate dehydrogenase (Nakamoto & Vennesland, 1960), like yeast glutathione reductase (Stern & Vennesland, 1960), exhibits B stereospecificity with pyridine nucleotides. A reaction mixture of 3.0 mL contained 24 μmol of sodium phosphate, pH 8.0, 135 μmol of [2-^{2}H]-glutamic acid titrated to pH 8.0–9.0 with NaOH, and 5.6 μmol of $NADP^+$. Glutamate dehydrogenase (~0.1 mL) in 50% glycerol was added and the reaction mixture kept overnight at 5 °C to allow the deuterium label to come to equilibrium. Enzyme assays demonstrated that glutamate dehydrogenase activity was still present after the 12-h incubation.

Glutamate dehydrogenase was then separated from the pyridine nucleotides by chromatography on a Sephadex G-50 column (1 × 22 cm) equilibrated with 10 mM sodium phosphate, pH 7.0, containing 3 mM EDTA. Fractions which absorbed light at 340 nm were pooled and applied to a Bio-Rad Cellex-T column (0.5 × 3 cm) which was equilibrated with the same buffer. After elution of $NADP^+$ (monitored by light absorbance at 260 nm) with 15 mM sodium phosphate, pH 7.0, the elution buffer was changed to 60 mM sodium phosphate, pH 7.6, containing 3 mM EDTA. Pure [^{2}H]NADPH was thus eluted directly in the standard buffer used for kinetic experiments. The purity of the product was checked by measuring the absorbance ratio A_{260}/A_{340}, which has a value of 2.4 for pure NADPH (Silverstein, 1965). All preparations of [^{2}H]NADPH used in this work had $A_{260}/A_{340} < 2.7$, corresponding to a sample purity exceeding 90%.

The effect of the deuterated substrate on the equilibrium constant of the glutamate dehydrogenase reaction was determined. The fractionation factor K_{eq}^{D}/K_{eq}^{H} had an observed value between 1.07 and 1.19 in favor of glutamate, in good agreement with the value of 1.18 reported by Schimerlik et al. (1975). Such a small change in the equilibrium constant should be negligible in terms of isotope enrichment of any particular substrate or product.

Preparation of Tetrahydro-NADP. This analogue was prepared by a modification of the method of Biellman & Jung (1971) for the preparation of tetrahydro-NAD. The repeated lyophilization called for in the original procedure resulted in considerable breakdown of the reduction product. Analysis by thin-layer chromatography and NMR spectroscopy indicated that the tetrahydro-NADP ($NADPH_4$) was being degraded to tetrahydronicotinamide and 2′-phosphoadenosine 5′-diphosphoribose (P-ADP-ribose). Thin-layer chromatography on silica gel in an ethanol–1 M ammonium acetate (6:4) solvent system resolved $NADPH_4$ from its contaminants. The

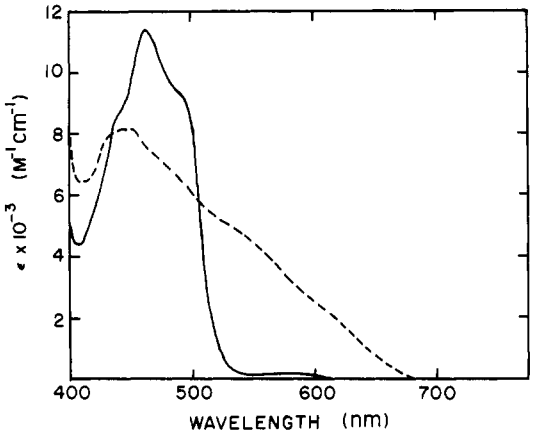

FIGURE 1: Spectra of the oxidized and two-electron reduced forms of glutathione reductase at 5 °C. Anaerobic spectra were recorded and plotted as extinction coefficient vs. wavelength. Samples were in 60 mM sodium phosphate, pH 7.6, containing 3 mM EDTA. (—) 7.6 μM enzyme-bound FAD, oxidized form; (- - -) 7.4 μM enzyme-bound FAD reduced by 160 μM NADPH.

mean R_f values determined for this system are as follows: P-ADP-ribose, 0.37; $NADPH_4$, 0.45; tetrahydronicotinamide, 0.74. The NMR spectrum of $NADPH_4$ shows three singlets in the region between 7 and 9 ppm. Signals at 8.50, 8.25, and 7.30 ppm correspond to protons at positions 2 and 8 of the adenine ring and position 2 of the pyridine ring of $NADPH_4$, respectively. The three signals yield an integration ratio of 1:1:1 for the pure product. The chemical shift for the olefinic proton of tetrahydronicotinamide is 7.50 ppm, and an apparent doublet between 7.3 and 7.5 ppm was indicative of partial breakdown of the reduction product. Consequently, once the catalytic reduction of $NADP^+$ was complete (as measured by the absorbance ratio A_{265}/A_{288}), the palladium-on-charcoal catalyst was removed from the reaction mixture by vacuum filtration through cellulose powder and then through a 0.45-μm Millipore filter. The product obtained by this method was greater than 90% pure as determined by thin-layer chromatography and NMR spectroscopy.

Results and Discussion

Reduction of Glutathione Reductase by NADPH. (1) Measurement of k_{obsd} for the Reductive Half-Reaction. When yeast glutathione reductase is reduced by an excess of NADPH, the resulting two-electron reduced enzyme binds one molecule of NADPH per enzyme-bound FAD with a dissociation constant of 2 μM (Bulger & Brandt, 1971b), to ultimately yield EH_2–NADPH rather than the free species EH_2 which is produced by reduction with NADH (Bulger & Brandt, 1971a) or reduced glutathione (GSH) (Massey & Williams, 1965). Reduction of the enzyme by NADPH is very rapid at 25 °C and is virtually complete within the dead time of the stopped-flow apparatus. In order to study the reductive sequence in detail, we performed the stopped-flow kinetic experiments described below at 5 °C. While the absorption spectrum of glutathione reductase has been shown to exhibit temperature-dependent shifts when measured by sensitive difference spectroscopy (Müller et al., 1973), the effects are generally small. In our hands, the spectra of the oxidized form of the enzyme measured at 5 and at 25 °C were essentially superimposable. Similarly, the spectrum obtained upon reduction of the enzyme by a 22-fold molar excess of NADPH at 5 °C was virtually superimposable upon the spectrum of the enzyme reduced by a 29-fold molar excess of NADPH at 25 °C. The spectra obtained at 5 °C are shown in Figure 1. These observations indicate the absence of major temperature-dependent conformational changes which might affect the integrity of the protein–flavin interactions and imply that the dissociation constant of the EH_2–NADPH complex is not radically different at the lower temperature.

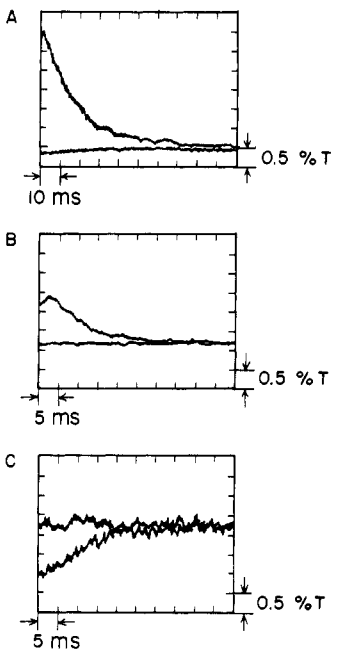

FIGURE 2: Tracing of stopped-flow oscilloscope traces of the transmittance changes which occur at 530, 420, and 675 nm after mixing oxidized glutathione reductase with NADPH at 5 °C. Both enzyme and NADPH were in 60 mM sodium phosphate, pH 7.6, containing 3 mM EDTA. (A) 530 nm, concentrations after mixing were 1.6 μM enzyme-bound FAD and 31 μM NADPH; (B) 420 nm, concentrations after mixing were the same as (A); (C) 675 nm, concentrations after mixing were 5.7 μM enzyme-bound FAD and 59 μM NADPH. In (A) and (B) the spectral band width was 0.90 nm, while in (C) it was 3.0 nm. For all three traces the noise-filter time constant was 0.5 ms. The horizontal trace in each oscilloscope trace was produced by manually triggering the oscilloscope after the first pass to display the $t = \infty$ transmittance.

Comparison of the spectra in Figure 1 of reactant (E) and product (EH_2–NADPH) at any wavelength allows one to predict the direction and magnitude of the absorbance change that should accompany the reductive half-reaction. One can then compare the absorbance change actually detected in the stopped-flow apparatus (i.e., the kinetically determined absorbance change) with this predicted change. Thus, at 530 nm an increase in absorbance occurs between $t \approx 5$ ms and $t = \infty$ which is approximately equal to the predicted increase. However, the predicted absorbance decrease at 462 nm is not seen in the stopped-flow apparatus, indicating that it is virtually complete within the dead time of the apparatus. In addition, at long wavelengths (650–730 nm) a decrease in absorbance is detected kinetically where one predicts either no change or an increase in absorbance, suggesting that the decay of an intermediate formed in the dead time of the apparatus is being observed. A further indication of the complexity of the reductive half-reaction is the observation of a kinetically detected absorbance increase rather than the predicted decrease at 440 nm.

In addition, while pseudo-first-order kinetics were observed at all wavelengths (the NADPH concentration was always at least 10 times that of the enzyme-bound FAD), the calculated values of k_{obsd}, the apparent first-order rate constant, were not uniform across the visible spectrum of the enzyme. The oscilloscope traces in Figure 2 illustrate this. It is apparent that the time course of the transmittance change at 530 nm is

Table I: Values of Observed Pseudo-First-Order Rate Constants for the Reaction of Glutathione Reductase with NADPH[a]

wavelength (nm)	NADPH concn (μM)	k_{obsd} (s^{-1})
420	16	102 ± 4
	27	117 ± 8
	53	132 ± 14
440	16	96 ± 6
462	15–53	*b*
505	16	53 ± 2
530	16	55 ± 2
	31	61 ± 2
	48	63 ± 4
650	56	137 ± 15
675	59	106 ± 12

[a] Experiments were performed in 60 mM sodium phosphate, pH 7.6, containing 3 mM EDTA at 5 °C. [b] In all cases the absorbance change at this wavelength was essentially complete within the dead time of the stopped-flow apparatus.

slower than that observed at 420 nm for the same NADPH concentration. The time course of the reaction monitored at 675 nm, however, is quite similar to that at 420 nm. The differences are more clearly seen by comparing the values of k_{obsd} shown in Table I: the rate constants evaluated from data at 420 and 440 nm are nearly identical and are quite similar to those obtained from data at long wavelengths (650 and 675 nm), but they are approximately twofold larger than the rate constants calculated from data at 505 and 530 nm for comparable NADPH concentrations. As noted above, the absorbance decreases in the region around 462 nm and the formation of the long-wavelength band were essentially complete within the dead time of the stopped-flow apparatus and therefore must be characterized by $k_{obsd} > 300\ s^{-1}$.

These results suggest that there is a minimum of three kinetically distinguishable steps in the reductive half-reaction. The first is a very rapid step completed within the dead time of the apparatus; the other two steps can be detected and studied by stopped-flow methods. The dependence of k_{obsd} on NADPH concentration was examined at 420 and 530 nm, wavelengths representative of spectral regions exhibiting different rate constants; these data are presented in the double-reciprocal plots of Figure 3. At 420 nm k_{obsd} has a limiting value of 153 s^{-1}, while at 530 nm the apparent first-order rate constant has a limiting value of 68 s^{-1}. The difference between the limiting values of k_{obsd} measured at 420 and 530 nm and the unequal values of the deuterium kinetic isotope effects on these two rate constants (see below) support the contention that they do represent separate steps of the reduction sequence. Within experimental error, first-order kinetic behavior [i.e., linear ln (fraction unreacted) vs. time plots] was observed at both 420 and 530 nm. While some coupling between the two kinetic processes might be expected, it was not detectable. This can be explained if (a) the process characterized by the absorbance change at 530 nm does not result in any significant further absorbance change at 420 nm and (b) the process monitored at 420 nm does not cause a significant absorbance change at 530 nm. It may be noted that the reduction of glutathione reductase by NADPH is kinetically more complex than the reduction of lipoamide dehydrogenase by the nonphysiological analogue, reduced 3-acetylpyridine adenine dinucleotide (AcPyADH), in which the initial, rapidly formed intermediate is converted to EH_2 without any other detectable intermediates (Matthews et al., 1979).

(2) *Spectrum of the Transient Enzyme Intermediate Formed within the Dead Time of the Stopped-Flow Spectrophotometer.* The spectrum of the transient intermediate

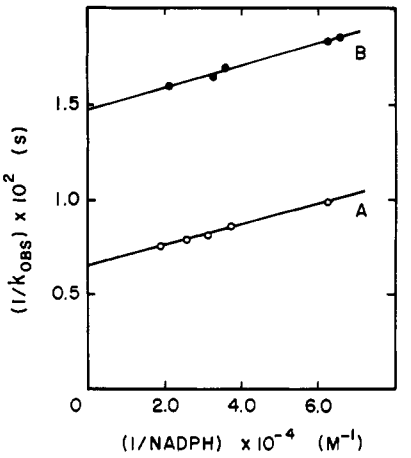

FIGURE 3: Plots of $1/k_{obsd}$ vs. 1/[NADPH] for the reduction of oxidized glutathione reductase by NADPH at 5 °C in 60 mM sodium phosphate, pH 7.6, containing 3 mM EDTA. Values of k_{obsd}, the pseudo-first-order rate constant, were determined as described under Experimental Procedures. Line A (○): 420 nm, enzyme-bound FAD concentration = 1.5 μM after mixing. Line B (●): 530 nm, enzyme-bound FAD concentration = 1.7 μM after mixing. The range of concentration of NADPH used was limited by the necessity of maintaining a 10-fold excess over enzyme-bound FAD concentration and the necessity of keeping the faster step slow enough to be within the accessible time range of the stopped-flow technique.

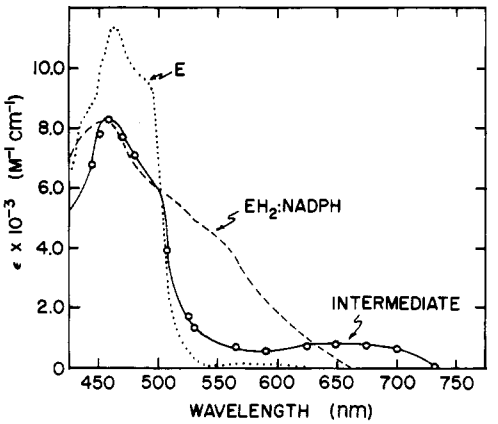

FIGURE 4: The spectrum of the transient intermediate produced during the deadtime of the stopped-flow spectrophotometer on mixing oxidized glutathione reductase with NADPH in 60 mM sodium phosphate, pH 7.6, containing 3 mM EDTA at 5 °C. The spectrum was constructed as described in the text and is the average of three experiments. It is plotted as extinction coefficient vs. wavelength. Shown for comparison are the spectrum of the oxidized form of the enzyme and the spectrum of the EH_2–NADPH complex.

observed immediately after mixing glutathione reductase with NADPH in the stopped-flow apparatus is compared with the spectra of E and EH_2–NADPH in Figure 4. The spectrum was calculated as described by Massey and co-workers (Massey et al., 1970; Walsh et al., 1973). The total absorbance change ($t \approx 5$ ms to $t = \infty$) observed on the oscilloscope was used to calculate $\Delta\epsilon$ at selected wavelengths from 420 to 730 nm. The absorbance at $t = \infty$ is assumed to be that of the final reduction product, EH_2–NADPH; ~90% of the enzyme will be in this form at the NADPH concentrations used in these experiments (Bulger & Brandt, 1971b). The difference $\epsilon_{EH_2\text{-NADPH}} - \Delta\epsilon$ at each wavelength was used to construct the spectrum of the transient intermediate. The prominent spectral characteristics of the intermediate are a broad, featureless absorbance band at long wavelengths and the partial bleaching of the flavin absorbance centered at 462 nm.

This spectrum resembles neither the neutral nor the anionic semiquinone forms of free (Beinert, 1956) or enzyme-bound

Table II: Kinetic Isotope Effects for Reduction of Glutathione Reductase Using (4S)-[^{2}H]NADPH

expt	source of NADPH[c]	NADPH concn (μM)	stopped-flow expt[a] wavelength (nm)	k_{obsd} (s^{-1})	k_{obsd}^H/k_{obsd}^D[d]	steady-state expt[b] ΔA_{340}/min	v_H/v_D
1	unlabeled, commercial	17	530	54 ± 5	1.0		
	unlabeled, enzymatic	21	530	54 ± 5			
2	unlabeled, commercial	27	530	61 ± 3	1.8		
	labeled, enzymatic	23	530	33 ± 2			
3	unlabeled, commercial	39	420	128 ± 17	1.0		
	unlabeled, enzymatic	39	420	123 ± 15			
4	unlabeled, commercial	31	420	122 ± 15	2.7		
	labeled, enzymatic	31	420	46 ± 3			
5	labeled, enzymatic	various	462	[e]			
6	unlabeled, commercial	53				0.210	1.7
	labeled, enzymatic	66				0.121	

[a] *Conditions: 60 mM sodium phosphate, pH 7.6, containing 3 mM EDTA at 5 °C.* [b] *Conditions: 60 mM sodium phosphate, pH 7.6,* containing 3 mM EDTA at 25 °C; GSSG concentration was 3.25 mM. [c] "Commercial" refers to commercially purchased NADPH, while "enzymatic" refers to NADPH prepared by the glutamate dehydrogenase method described under Experimental Procedures. [d] Where necessary, the rate constant for unlabeled NADPH was corrected to that of the concentration of labeled NADPH by using the concentration dependence illustrated in Figure 3. [e] In all cases the absorbance change at this wavelength was essentially complete within the dead time of the stopped-flow apparatus.

(Massey et al., 1970) flavin, which do absorb light at wavelengths above 500 nm. Nor does it exhibit properties which correspond to covalent flavin analogues substituted at various positions of the isoalloxazine ring (Hemmerich & Jorns, 1972).

The spectrum of the rapidly formed intermediate shown in Figure 4 does have features comparable to the spectra of intermediates detected during the pyridine nucleotide dependent reduction of other flavoenzymes (Massey et al., 1970). Massey & Ghisla (1974) suggest that these intermediates are charge-transfer complexes between the oxidized flavin and the reduced pyridine nucleotide, but data firmly establishing that electron transfer has not occurred were not presented. However, studies with the pyridine nucleotide analogue AcPyADH reveal that in the case of both lipoamide dehydrogenase (Matthews et al., 1979) and melilotate hydroxylase (Schopfer & Massey, 1979) similar spectra can be observed prior to hydride transfer to the enzyme flavin. The spectrum of the transient intermediate observed during the reduction of glutathione reductase is also similar to the static spectra of charge-transfer complexes formed when the oxidized form of Old Yellow enzyme binds any of a number of para-substituted phenols (Abramovitz & Massey, 1976).

Several model systems designed to study hydride transfer from pyridine nucleotide to the isoalloxazine ring also exhibit preequilibrium complexes showing increased absorbance at long wavelengths (Porter et al., 1973; Blankenhorn, 1975a,b, 1976). These complexes, which form too rapidly to be seen by stopped-flow techniques, precede and lead directly to the reduced flavin species. The extinction coefficients of these long-wavelength bands range from 1000 (Porter et al., 1973) to 600 M^{-1} cm^{-1} (Blankenhorn, 1975a). For the transient glutathione reductase intermediate (Figure 4), the extinction coefficient of the long-wavelength transition is calculated to be 700–900 M^{-1} cm^{-1}. The experimental evidence, therefore, suggests that the transient intermediate whose spectrum is shown in Figure 4 may represent a rapidly formed preequilibrium charge-transfer complex between the oxidized flavin of glutathione reductase and NADPH.

The alternative possibility that the transient intermediate is a complex between reduced flavin and $NADP^+$ is rejected on the basis of kinetic isotope effect experiments described in the next section and on the basis of spectroscopic data reported by Massey & Williams (1965). Massey & Williams prepared the four-electron reduced form of glutathione reductase (in which both the FAD and the redox-active protein disulfide are reduced) in the presence of sodium arsenite and $NADP^+$. The spectrum of the resulting EH_4–$NADP^+$ complex, in which the nascent active-site dithiol is presumably combined with arsenite, clearly differs from that shown in Figure 4. The comparison must be viewed with some caution, however, due to the presence of arsenite for the spectrum of EH_4–$NADP^+$.

Reduction of Glutathione Reductase by (4S)-[2H]NADPH. (1) Stopped-Flow Studies. Table II summarizes a series of kinetic experiments in which the rate of reduction of glutathione reductase by (4S)-[^{2}H]NADPH was measured. As a control, unlabeled NADPH was prepared by the glutamate dehydrogenase method with unlabeled glutamic acid as the substrate. The data in Table II at both 420 (experiment 1) and 530 nm (experiment 3) show that the NADPH prepared enzymatically yields the same rate constants as commercial NADPH. Therefore, the method of preparing deuterated NADPH does not introduce artifacts which affect the observed rate constants.

With [^{2}H]NADPH, the partial bleaching of the flavin absorbance centered around 462 nm remains virtually complete within the dead time of the stopped-flow apparatus. However, the apparent rate constants at both 420 and 530 nm exhibit isotope effects. Furthermore, the isotope effects are different, providing additional evidence that the rate constants measured at these wavelengths represent two different steps of the reaction scheme. Thus the results with [^{2}H]NADPH corroborate the three-step minimal mechanism for the reductive half-reaction.

The value of k_{obsd}^H/k_{obsd}^D measured at 420 nm in several experiments ranged from 2.4 to 2.9, with an average value of 2.7, in reasonable agreement with the values of isotope effects for flavin reduction in a number of model (Suelter & Metzler, 1960; Porter et al., 1973) and biological systems (Schopfer & Massey, 1979; Strittmatter, 1962). Perhaps most significantly, the reduction of lipoamide dehydrogenase by (4S)-[^{2}H]AcPyADH also exhibits a kinetic isotope effect of 2.6 (Matthews et al., 1979). It may be noted that larger values of k^H/k^D have been observed occasionally when a nonphysiological pyridine nucleotide (e.g., AcPyADH or PyAlADH) has been used as the electron donor (Schopfer & Massey, 1979; Strittmatter, 1966).

The agreement between the kinetic isotope effect observed at 420 nm with glutathione reductase and the isotope effects detected in other flavin systems is strong evidence that this particular absorbance change represents oxidation of NADPH

and, hence, reduction of the enzyme. The long-wavelength absorbance centered at 675–700 nm decays with virtually the same rate constant as that observed at 420 nm, suggesting that oxidation of NADPH accompanies the disappearance of the long-wavelength band, in agreement with its assignment to a charge-transfer complex between reduced pyridine nucleotide and oxidized flavin.

Interpretation of the 1.8-fold change in the apparent rate constant at 530 nm is not as direct. The increased absorbance of EH_2 between 500 and 600 nm, relative to the oxidized enzyme, has been ascribed to a complex between one of the nascent thiols, generated upon reduction of the enzyme active site, and the oxidized flavin (Abramovitz & Massey, 1976; Matthews & Williams, 1976). Accordingly, the 530-nm absorbance change likely represents electron transfer from flavin to the redox-active disulfide bond and/or formation of the thiol–flavin complex (possibly from a covalent adduct at C-4a of the isoalloxazine?) and might not be expected to exhibit a kinetic isotope effect. Inspection of Table II reveals that k_{obsd} measured at 420 nm with [^{2}H]NADPH is smaller than the limiting value of 68 s^{-1} for the 530-nm step. Thus, when the enzyme is reduced by [^{2}H]NADPH, hydride transfer from bound NADPH to the enzyme becomes rate limiting. The succeeding step observed at 530 nm cannot occur more quickly than the step at 420 nm, resulting in an apparent kinetic isotope effect. This interpretation is supported by a computer analysis of the kinetics of reduction described below.

(2) Steady-State Turnover with [^{2}H]NADPH. Table II also shows the effect of (4*S*)-[^{2}H]NADPH on the steady-state turnover of glutathione reductase at 25 °C. The initial velocities show a kinetic isotope effect of 1.7, similar to the isotope effect on k_{obsd} measured at 530 nm, which is the rate-limiting step in the reductive half-reaction as detected by stopped-flow techniques. The 1.7-fold decrease in the steady-state turnover must arise, then, from the intrinsic isotope effect on hydride transfer. These results establish that the reductive half-reaction is rate-limiting overall in catalysis with glutathione reductase. This contrasts with lipoamide dehydrogenase where reoxidation of EH_2 by lipoamide is rate-limiting in the overall reaction (Matthews et al., 1979) and represents one of the few significant differences in the kinetics of these two closely similar flavoenzymes.

Interaction of Glutathione Reductase with 1,4,5,6-Tetrahydronicotinamide Adenine Dinucleotide Phosphate. The preceding results suggest that one intermediate in the reductive half-reaction is a charge-transfer complex between NADPH and the oxidized enzyme flavin. An alternative possibility is that NADPH binding induces a protein conformational change which moves an amino acid side chain (e.g., a tyrosine phenolic group) into a transient charge-transfer interaction with the isoalloxazine ring, generating the spectrum in Figure 4. Since the intermediate is formed prior to hydride transfer from NADPH, the binding of appropriate NADPH analogues might induce a similar spectral change if the alternative explanation were correct.

The visible absorption spectrum of glutathione reductase is not detectably altered when E binds $NADP^+$, but $NADP^+$ is not a very satisfactory analogue of NADPH. The positive charge on the nicotinamide ring can change the way the nicotinamide moiety interacts with enzymes. Thus, phenyl adenine dinucleotide which lacks a positive charge acts as an NADH analogue rather than as an NAD^+ analogue in binding to alcohol dehydrogenase (Danenberg et al., 1978). $NADPH_4$, while not functional as a reductant, would appear to be a good analogue of NADPH; the formal electronic structure and size

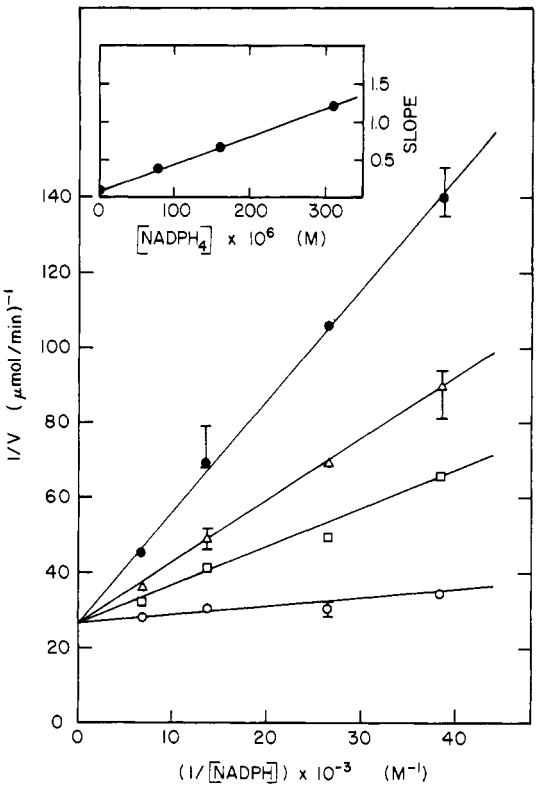

FIGURE 5: Double-reciprocal plot demonstrating that $NADPH_4$ is a competitive inhibitor of glutathione reductase with respect to NADPH. The steady-state kinetics were run at 25 °C in 60 mM sodium phosphate, pH 7.6, containing 3 mM EDTA. The concentrations of $NADPH_4$ were (○) none, (□) 81, (△) 163, and (●) 325 μM. Inset: Secondary plot of the slopes of the primary plot vs. $NADPH_4$ concentration.

of the two molecules are the same. Figure 5 shows that $NADPH_4$ is a linear competitive inhibitor with respect to NADPH, with $K_i = 16 \pm 3$ μM. This compares with the K_m for NADPH of 4–13 μM (Massey & Williams, 1965; Mavis & Stellwagen, 1968; Moroff & Brandt, 1973), while the K_i for $NADP^+$ as a competitive inhibitor is 70 μM (Bulger & Brandt, 1971b). The lower K_i of $NADPH_4$ relative to $NADP^+$ supports the contention that it is a better analogue of NADPH.

The absorption spectrum of 8.6 μM enzyme-bound FAD in the presence of 156 μM $NADPH_4$ was indistinguishable from that of the free enzyme. At this $NADPH_4$ concentration, 91% of the enzyme should exist as the E–$NADPH_4$ complex, when $K_d = 16$ μM as obtained in the inhibition experiments was used. Furthermore, difference spectroscopy revealed no detectable changes in the absorption spectrum of the enzyme between 400 and 700 nm. To the extent that $NADPH_4$ binding mimics NADPH binding, we conclude that the intermediate spectrum shown in Figure 4 is not a consequence of a conformational change induced when reduced pyridine nucleotide binds. Since $NADPH_4$ has only two π electrons in the single double bond of the tetrahydronicotinamide ring, it also should not be expected to function as a donor in a charge-transfer interaction with the enzyme-bound flavin.

Computer Simulation of the Glutathione Reductase Reductive Half-Reaction. Equation 1 is a minimal kinetic

$$A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} B \xrightarrow{k_3} C \xrightarrow{k_4} D \qquad (1)$$

scheme which accommodates the experimental data presented above. In this scheme, A represents the oxidized form of glutathione reductase which is in rapid equilibrium with B,

the enzyme–substrate complex E–NADPH. The pseudo-first-order rate constant k_1 is equal to k'[NADPH], where k' is the bimolecular rate constant for combination of NADPH with E. C is a reduced enzyme species formed in the process monitored at 420 nm in which hydride transfer from NADPH to enzyme occurs, and k_3 is the rate constant for this step. D represents the final product of the half-reaction, the EH_2–NADPH complex. The rate constant k_4 corresponds to the process observed at 530 nm in the stopped-flow apparatus. Equation 1 is a simple kinetic model and as such does not specifically identify the molecular nature of C, nor does it include specific steps for the dissociation of $NADP^+$ or for the binding of NADPH to EH_2 to yield EH_2–NADPH, although these two steps clearly must occur. No experimental observations can be identified with either of them, but the binding of NADPH to EH_2 has been shown to be very rapid (Bulger & Brandt, 1971b). The two kinetically detectable steps also cannot be unequivocally correlated with discrete molecular species. Species with spectra comparable to reduced flavin or to a covalent thiolate–flavin 4a adduct are not detected, although they are plausible intermediates in electron transfer from NADPH to protein disulfide (Matthews et al., 1979). It is possible that electron transfer is relatively concerted so that discrete intermediates are not detected, as suggested by Matthews et al. (1979) for lipoamide dehydrogenase. What is clear is that formation of EH_2 with its characteristic absorbance band at 530 nm is at least a three-step process in glutathione reductase.

The adequacy of the kinetic model in eq 1 to accommodate the data reported in this paper has been tested in two ways. Equations can be derived (Benson, 1960) for the concentrations of A, B, C, and D as functions of time. A computer program has been written which uses values for k_1, k_2, k_3, and k_4 to calculate the concentrations of C and D at selected times. It then computes by linear regression apparent rate constants from the slopes of plots of log $(([A]_0 - [D])/[A]_0)$ vs. time ($2.3 \times \text{slope} = -k_{530}^{app}$) and of log $(([A]_0 - [C] - [D])/[A]_0)$ vs. time ($2.3 \times \text{slope} = -k_{420}^{app}$). These predicted values of k_{420}^{app} and k_{530}^{app} were then compared with the experimentally obtained values. Second, we have tested the adequacy of the explanation for the 1.8-fold apparent isotope effect detected at 530 nm.

Values for the rate constants were assigned as follows. Values for $k_3 = 153\ s^{-1}$ and $k_4 = 68\ s^{-1}$ were taken from the limiting values of k_{obsd} measured at 420 and 530 nm, respectively. The abscissa intercept of the line through the 420 nm data in Figure 3 yields the dissociation constant $K_d = k_2/k' = 8.3\ \mu M$ for the rapidly formed intermediate (Strickland et al., 1975). Assuming k' measures a diffusion-controlled process, a value in the range 10^7–$10^8\ M^{-1}\ s^{-1}$ can be assigned to k' (Hammes, 1968). Varying k' had little effect on the predicted values of k_{420}^{app} and k_{530}^{app}. Selection of values for k' and [NADPH] fixes k_1 and k_2. To explore the kinetic isotope effect, we decreased k_3 by a factor of 2.7 (the kinetic isotope effect at 420 nm) to a value of $56\ s^{-1}$; the value of k_4 was left at $68\ s^{-1}$.

Examination of the results of these tests in Table III indicates that eq 1 represents a satisfactory minimal kinetic mechanism. For the particular case shown, with 31 μM NADPH the predicted values of k_{420}^{app} and k_{530}^{app} agree satisfactorily. It is also clear that decreasing k_3 by 2.7-fold from 153 to $56\ s^{-1}$ not only leads to the prediction that k_{420}^{app} will decrease 2.4- to 2.7-fold but also leads to the prediction that k_{530}^{app} should decrease 1.7-fold. This agrees with the experimental kinetic isotope effect measured at 530 nm and supports the explanation that it results entirely from the k_3 step having become rate limiting when [^{2}H]NADPH is used as the substrate. It is not necessary to propose that the value of k_4 is affected by substitution of deuterium in NADPH.

Table III: Comparison of Apparent Rate Constants Predicted by Equation 1 with Experimental Data

substrate	wavelength (nm)	exptl[a] k_{obsd} (s^{-1})	k_λ^{app} calcd for eq 1[b] (s^{-1})
NADPH	420	122 ± 15	105–117
[^{2}H]NADPH	420	46 ± 3	43–44
NADPH	530	61 ± 3	62–64
[^{2}H]NADPH	530	34 ± 2	36–37

[a] The experimental data are from an experiment in which 5 μM glutathione reductase was mixed in the stopped-flow apparatus with an equal volume of 62 μM reduced pyridine nucleotide at 5 °C in 60 mM sodium phosphate, pH 7.6, containing 3 mM EDTA. [b] These values were calculated as described in the text by using $[A]_0 = 2.5\ \mu M$, $k_3 = 153\ s^{-1}$ for reactions with NADPH or $56\ s^{-1}$ for reactions with [^{2}H]NADPH, $k_4 = 68\ s^{-1}$, and $k_2/k' = 8.3\ \mu M$, where $k' = k_1/[NADPH]$. The range of values indicated in this column reflects the range of values chosen for k_1 which was 250–$1000\ s^{-1}$.

Conclusion

It is well documented that the isoalloxazine ring can participate in charge-transfer complexes with a variety of ligands (Tamaru & Ichikawa, 1975; Foster, 1969; Massey & Ghisla, 1974). Depending on its oxidation state, it can act either as a donor or as an acceptor. A number of model systems have been used to study hydride transfer from reduced pyridine nucleotide to flavins or other heterocyclic conjugated molecules (Porter et al., 1973; Blankenhorn, 1975a,b, 1976; Bruice et al., 1971; Creighton et al., 1973; Kurz & Frieden, 1975; Hajdu & Sigman, 1976; Steffens & Chipman, 1971), and kinetic and thermodynamic data suggest that a kinetically significant complex forms between the reactants prior to hydride transfer. In some cases (Porter et al., 1973; Blankenhorn, 1975a,b, 1976; Hajdu & Sigman, 1976) spectral evidence comparable to that presented here has identified the intermediate as a charge-transfer complex. It appears that these model systems are accurate and valuable prototypes. The fact that charge-transfer interactions seem to precede hydride transfer not only in model systems but also in an enzyme system suggests that the transition state for flavin reduction involves extensive transfer of negative charge. In support of this idea, linear free energy relationships for the NADH-dependent reduction of a series of para-substituted 2,6-dinitrobenzenesulfonates yield ρ values which compare well with other processes in which there is substantial transfer of charge in the transition state (Kurz & Frieden, 1975). In pyridine nucleotide dependent reduction of flavins (e.g., glutathione reductase), charge-transfer interactions may produce a metastable complex which is structurally related to the transition state of the actual electron transfer step and thereby facilitate that step.

In this regard, the preequilibrium complex observed during reduction of lipoamide dehydrogenase by AcPyADH (Matthews et al., 1979) shows no major perturbation of the flavin absorption peak near 450 nm, suggesting that charge transfer is not optimal. This may explain why electron transfer from this analogue to the enzyme is so slow ($k = 33\ s^{-1}$ at 25 °C). One might predict that the more rapid reduction of lipoamide dehydrogenase by NADH proceeds via a preequilibrium complex in which the spectrum of the flavin peak near 450 nm is significantly perturbed.

Finally, the data show that the last detectable step of the reductive half-reaction (formation of the thiolate to flavin

charge-transfer complex) is rate limiting overall in catalysis by glutathione reductase. Previous evidence summarized by Williams (1976) suggested that reoxidation of EH_2 by GSSG was rate limiting in the overall reaction. The results presented here contradict that conclusion and support the conclusions drawn from more recent stopped-flow kinetic studies of the reoxidation of the two-electron reduced form of glutathione reductase by GSSG, yielding a limiting rate constant of 1200 s^{-1} for the oxidative half-reaction (Pyne, 1976).

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