1785–1788.
Hoffmann, P. J., & Blakley, R. L. (1975) *Biochemistry 14,* 4804–4812.
Imai, K., Fujii, S., Takanohashi, K., Furukawa, Y., Masuda, T., & Honjo, M. (1969) *J. Org. Chem. 34,* 1547–1550.
Jain, T. C., Russel, A. F., & Moffatt, J. G. (1973) *J. Org. Chem. 38,* 3179–3186.
King, C. D., & Lancker, J. L. V. (1969) *Arch. Biochem. Biophys. 129,* 603–608.
Langer, L., & Klenow, H. (1960) *Biochim. Biophys. Acta 37,* 33–37.
Larsson, A. (1969) *Eur. J. Biochem. 11,* 113–121.
Maley, G. F., & Maley, F. (1961) *J. Biol. Chem. 235,* 2964–2967.
Moffat, J. G., & Khorana, H. G. (1971) *J. Am. Chem. Soc. 83,* 649–658.
Moore, E. C. (1967) *Methods Enzymol. 12A,* 155–164.
Moore, E. C., & Hurlbert, R. B. (1966) *J. Biol. Chem. 214,* 4802–4809.
Moore, E. C., Booth, B. A., & Sartorelli, A. C. (1971) *Cancer Res. 31,* 235–238.
Moore, E. C., Peterson, D., Yang, L. Y., Yeung, C. Y., & Neff, N. F. (1974) *Biochemistry 13,* 2904–2907.
Morris, N. R., & Fischer, G. A. (1960) *Biochim. Biophys. Acta 42,* 183–184.
Robins, M. J., & Muhs, W. H. (1976) *J. Chem. Soc., Chem. Commun.* 269.
Robins, M. J., McCarthy, J. R., Jr., Jones, R. A. & Mengel, R. (1973) *Can. J. Chem. 51,* 1313–1321.
Segal, I. H. (1975) *Enzyme Kinetics,* p 227 ff, Wiley, New York, N.Y.
Singh, D., Tamao, Y., & Blakley, R. L. (1977) *Adv. Enzyme Regul. 15,* 81–100.
Suhadolnik, R. J. (1970) *Nucleoside Antibiotics,* pp 298–389, Wiley, New York, N.Y.
Suhadolnik, R. J., Finkel, S. I., & Chassy, B. M. (1968) *J. Biol. Chem. 243,* 3532–3537.
Turner, M. K., Abrams, R., & Liebeman, I. (1968) *J. Biol. Chem. 243,* 3725–3728.
Vitols, E., Brownson, C., Gardiner, M., & Blakley, R. L. (1967) *J. Biol. Chem. 242,* 3035–3041.
Weith, H. L., Wiebers, J. L., & Gilham, P. J. (1970) *Biochemistry 9,* 4396–4401.
Whittle, E. D. (1966) *Biochim. Biophys. Acta 114,* 44–60.
Yoshikawa, M., Kato, T., & Takenishi, T. (1967) *Tetrahedron Lett. 50,* 5065–5068.
Young, C. W., & Hodas, S. (1964) *Science 146,* 1172–1174.
Young, C. W., Schochetman, G., & Karnofsky, D. A. (1967) *Cancer Res. 27,* 526–534.

Kinetics of Ligand Binding to Dihydrofolate Reductase: Binary Complex Formation with NADPH and Coenzyme Analogues†

Susan M. J. Dunn, John G. Batchelor,‡ and Rodney W. King*

ABSTRACT: The reaction between dihydrofolate reductase from *L. casei* MTX/R and NADPH or its analogues has been investigated by stopped-flow fluorescence techniques. The formation of the enzyme–coenzyme complex is characterized by quenching of the near ultraviolet fluorescence of the enzyme and enhancement of the fluorescence of the NADPH chromophore. These spectral changes take place in two phases: the faster characterized by an apparent bimolecular rate constant near 10^7 M^{-1} s^{-1}, the slower by a unimolecular rate constant whose value is dependent on pH and varies from 0.06 to 0.012 s^{-1}. The relative amplitudes of the fast and slow phases are also pH dependent, the fast change dominating at low pH. There is no direct coupling of the fast and slow reactions, since the slow phase appears in the reaction trace only after the fast phase has been saturated. These results suggest that the enzyme exists in at least two interconvertible forms whose relative proportions are pH dependent. NADPH appears to bind rapidly and exclusively to one of these forms, the slow phase probably being a reflection of the interconversion of the forms followed by rapid coenzyme binding. The fast association rate is dependent on the viscosity of the medium and has an enthalpy of activation of only 2.5 kcal mol^{-1}, suggesting that the association is diffusion controlled. The binding of deamino-NADPH and etheno-NADPH follows the same kinetic pattern as that of NADPH; however, the ligands 3-acetylpyridine-NADPH, thionicotinamide-NADPH, and $NADP^+$ exhibit a third phase of relatively small amplitude and intermediate rate in the association reaction. It is proposed that in these cases the coenzyme analogues can bind to either form of the enzyme and that the intermediate rate reflects the interconversion of the binary complexes.

Dihydrofolate reductase (5,6,7,8-tetrahydrofolate:$NADP^+$ oxidoreductase, EC 1.5.1.3) catalyzes the NADPH-dependent reduction of 7,8-dihydrofolate (DHF) to 5,6,7,8-tetrahydrofolate (THF). The enzyme is therefore necessary for the formation of THF and its derivatives which are essential cofactors in the important metabolic reactions involving transfer of one-carbon units. In conjunction with thymidylate synthetase, the enzyme is essential for thymidylate biosynthesis and hence for DNA synthesis.

The enzyme has been the subject of extensive study (reviewed by Blakley, 1969) not only because of its importance in intermediary metabolism but also because it is the target

† From the National Institute for Medical Research, London NW7 1AA, Great Britain. *Received November 3, 1977; revised manuscript received March 3, 1978.* S.M.J.D. holds an MRC research studentship.
‡ Present address: Medical Computing Unit, Charing Cross Hospital, London W6 8RF, Great Britain.

0006-2960/78/0417-2356$01.00/0

enzyme of a group of chemotherapeutic agents which are widely used as antibacterial and antitumor agents.

The enzyme used in this study of the binding of coenzyme and coenzyme analogues was obtained from a methotrexate-resistant strain of *Lactobacillus casei* (*L. casei* MTX/R). Its preparation, isolation, and physical properties have been fully described (Dann et al., 1976).

Understanding the binding of coenzyme to the enzyme is an important prerequisite to understanding the binding of inhibitor molecules to the enzyme–coenzyme complex.

Kinetic experiments on the binding of coenzymes to preformed enzyme–inhibitor complexes and of inhibitor to enzyme–coenzyme complexes indicate that the binding mechanism to form ternary enzyme–coenzyme–inhibitor complexes is not ordered (Dunn & King, unpublished results; cf. Blakley et al., 1971). However, the binding of inhibitors to the binary enzyme–coenzyme complex is much stronger than to the enzyme alone, as is the binding of coenzyme to the preformed enzyme–inhibitor binary complex.

The binding properties of the enzyme are therefore strongly influenced by the presence or absence of a complementary ligand.

This paper describes the kinetic events during formation of binary enzyme–coenzyme complexes and attempts to assess the importance of alterations to the chemical structure of the coenzyme.

Experimental Section

Materials: Enzyme. The isolation and purification of dihydrofolate reductase from *L. casei* MTX/R has been described by Dann et al. (1976). The enzyme used in this investigation was stored at −15 °C in lyophilized form until required.

Coenzymes. 3-Acetylpyridine-$NADP^+$, deamino-NADPH, $NADP^+$, NADPH, and thionicotinamide-$NADP^+$ were obtained from commercial sources. The method used routinely for the reduction of 3-acetylpyridine-$NADP^+$ and thionicotinamide-$NADP^+$ was as follows.

Five milligrams of coenzyme was dissolved in 1.8 mL of 50 mM Tris-Cl buffer, pH 8.0. Seven milligrams of DL-isocitrate, 40 μL of $MnCl_2$ (40 mg/mL), and 0.2 mL of pig heart isocitrate dehydrogenase (10 mg/mL) were added. After mixing the solution was shielded from light and left for approximately 16 h at 4 °C. The mixture was then applied to a 5 × 2 cm column of DE-52 cellulose preequilibrated in 50 mM Tris, pH 8.0. After extensive washing with the same buffer, the column was eluted with a gradient formed from 60 mL of 50 mM Tris, pH 8.0, and 60 mL of 50 mM Tris, 300 mM KCl, pH 8.0. The concentration of reduced coenzyme eluted could be determined from its published extinction coefficient at 360 nm (for 3-acetylpyridine-NADPH) or 395 nm (for thionicotinamide-NADPH) (P-L Biochemicals Inc., 1973). The reduced coenzymes could be stored in solution at pH 8.0 and 4 °C for at least 4 weeks without detectable decomposition.

1,N^6-Ethenoadenine-NADPH was prepared as described by Neef & Huennekens (1976).

The purity of the coenzymes has been checked using paper chromatography. The technique and solvent systems II, III, and VI described in Circular OR-18 (P-L Biochemicals Inc., 1973) were used. The coenzymes were visualized either by visible fluorescence, by fluorescence quenching, or by staining (Wade & Morgan, 1953; Hanes & Isherwood, 1949). Impurities were detected in two of the coenzymes. A faintly fluorescent spot moving behind the main spot in system III and ahead of the main spot in system VI contaminated the 3-acetylpyridine-NADPH. This spot did not correspond to any other of the coenzymes. A very faint fluorescent spot in system VI, not seen in the other two solvent systems and also not corresponding to any of the other coenzymes, contaminated the etheno-NADPH. The level of these contaminants, as judged by their fluorescence and staining, was very low, and we feel is most unlikely to influence the results.

Equilibrium Dissociation Constants. Equilibrium dissociation constants for dihydrofolate reductase–coenzyme complexes were determined by fluorescence titrations at 25 °C using a Farrand spectrofluorimeter with excitation and emission wavelengths of 290 nm and 340 nm, respectively. Small relative volumes of ligand were added to 2.5 mL of dihydrofolate reductase solution contained in a 1-cm^2 cuvette. Standard tryptophan solutions were used to correct for light absorption by added ligands. Equilibrium fluorescence readings were recorded 5 min after ligand addition, and dissociation constants were calculated by the nonlinear regression technique described below.

Kinetic Measurements. Kinetic data were obtained using a Durrum D-110 (Durrum Instrument Corp., Calif.) stopped-flow apparatus operating in the fluorescence mode. Complex formation was followed by using an excitation wavelength of 290 nm and monitoring either the quenching of the protein intrinsic fluorescence using a 341-nm interference filter or, where appropriate, the enhancement of coenzyme fluorescence by energy transfer (Velick, 1958) using a 449-nm interference filter. A Wood's glass filter on the excitation side removed stray light above 400 nm. Signal smoothing was obtained by using a quartz-plate beam splitter in the incident beam reflecting approximately 5% of the incident light on to a photodiode. The amplified photodiode output and the photomultiplier output were fed into a source-ratioing amplifier (Anson & Bayley, 1976). Further smoothing of the amplifier output was obtained by using appropriate electronic filtering. Reaction traces were collected by passing the smoothed and amplified signal into a 200-point signal averager for temporary storage prior to data transfer to the disc of an HP3000 computer. The collected fluorescence data were simultaneously displayed on an oscilloscope. In most experiments the sum of at least four experimental runs was used for data analysis.

All experiments were carried out at 25 °C unless otherwise stated. Buffer systems used were: pH 5.25–5.5, 15 mM sodium acetate, 500 mM KCl; pH 5.5–7.5, 15 mM BisTris, 500 mM KCl; pH 7.5–8.0, 15 mM Tris, 500 mM KCl.

Data Analysis. All data were analyzed by nonlinear regression using computer programs which are described in detail elsewhere)j. G. Batchelor, manuscript in preparation). These programs use the algorithm of Marquardt (1963) which combines the Gauss–Newton and steepest descent methods of minimizing the sum of squares of residuals. The statistical output included standard errors of the best fit parameter values estimated using the assumption of a linear model about the solution. In all cases plots were obtained of the data and the best fit curve, to aid in the assessment of the fit of the data to the model.

Kinetic data were analyzed using either a single exponential model

$$F(t) = \mathrm{AMP}_1 \exp(-k_1 t) + \text{baseline}$$

or a double exponential model

$$F(t) = \mathrm{AMP}_1 \exp(-k_1 t) + \mathrm{AMP}_2 \exp(-k_2 t) + \text{baseline}$$

where $F(t)$ is the observed fluorescence signal, the ks are rate constants and t is time.

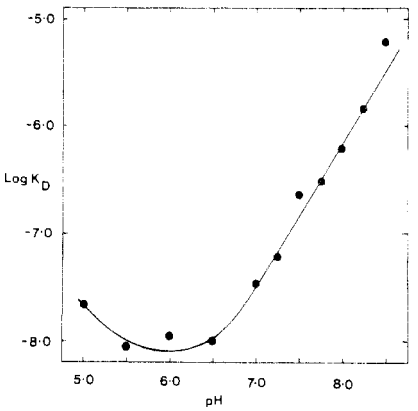


FIGURE 1: Variation in K_d with pH for NADPH and dihydrofolate reductase. Measurements made at 25 °C.

Binding curves were analyzed using the model

$$F = F(\mathrm{L})L_T + F(\mathrm{E})E_T + (F(\mathrm{C}) - F(\mathrm{L}) - F(\mathrm{E}))[\mathrm{C}]$$

given that $[\mathrm{C}] = B - SQRT(B^2 - E_T L_T)$ and

$$B = (L_T + E_T + K_d)/2$$

where F is the observed fluorescence, $F(\mathrm{L})$, $F(\mathrm{E})$, and $F(\mathrm{C})$, are the fluorescence yields per unit concentration of ligand, enzyme and complex, respectively, L_T is the total concentration of ligand, E_T is the total concentration of enzyme, [C] the concentration of enzyme-ligand complex, and K_d the ligand dissociation constant. Thus any dilution caused by addition of ligand and any depletion of free ligand concentration caused by binding to the enzyme are accounted for.

Values of pKs were determined using the model

$$F = F_1 + (F_2 - F_1)\left(\frac{10^{-pK}}{10^{-pK} + 10^{-pH}}\right)$$

where F_1 is the property observed at low pH and F_2 the same property observed at high pH.

Results

Fluorimetric Measurements of the Interaction of Dihydrofolate Reductase with Coenzymes. When dihydrofolate reductase was excited at 290 nm, the fluorescence emission spectrum showed a maximum at 340–350 nm, typical of the fluorescence spectra reported for other proteins (Udenfriend, 1962). The addition of NADPH or any of the other coenzymes examined quenched this fluorescence (see Table I). In the case of the reduced coenzymes, i.e., NADPH, deamino-NADPH, etheno-NADPH, thionicotinamide-NADPH, and 3-acetylpyridine-NADPH, formation of the binary complex with the enzyme resulted in the appearance of a new emission maximum at 420–450 nm, characteristic of activation by energy transfer, described by Velick (1958) for lactate and glyceraldehyde-3-phosphate dehydrogenases. Under identical conditions, binding to the enzyme caused a greater intensification of the energy-transfer fluorescence of 3-acetylpyridine-NADPH than of NADPH. A similar differential effect for the binding of these coenzymes to beef heart lactate dehydrogenase has been reported (Shifrin & Kaplan, 1958).

The quenching of the enzyme fluorescence at 340 nm was used to determine the dissociation constants of the various enzyme–coenzyme complexes and the results of such experiments performed at pH 6.0 are shown in Table I.

Dissociation constants of a number of binary complexes have been examined over a pH range of 5.0 to 8.5 and the results show that, although tightest binding occurs at pH 6.0, the variation between pH 5.0 and pH 7.0 is only slight, whereas the dissociation constants determined at pH values above 7.0 were considerably increased and the maximum quench was reduced. This effect is particularly marked for NADPH as ligand (Figure 1).

TABLE I: Dissociation Constants and Fluorescence Quenching Properties of Various Coenzyme–Dihydrofolate Reductase Complexes.[a]

Coenzyme	K_d (M)	Maximum quench (%)
NADPH	$(1.0 \pm 0.1) \times 10^{-8}$	88
Deamino-NADPH	$(3.2 \pm 0.1) \times 10^{-8}$	83
Etheno-NADPH	$(3.2 \pm 0.3) \times 10^{-8}$	95
3-Acetylpyridine-NADPH	$(4.7 \pm 0.2) \times 10^{-7}$	85
Thionicotinamide-NADPH	$(3.7 \pm 0.2) \times 10^{-6}$	60
$NADP^+$	$(1.2 \pm 0.1) \times 10^{-5}$	65

[a] Experiments carried out at pH 6.0 and 25 °C.

Evidence from molecular weight determinations by column chromatography and ultracentrifugation (Dann et al., 1976) and from solubility studies (Dann, 1975) suggest that the enzyme is aggregated at low ionic strength. However, this aggregation appears to be abolished in NMR experiments at enzyme concentrations approaching 1.0 mM by 0.5 M KCl present in solution (Roberts et al., 1974). We have therefore included 0.5 M KCl in all our buffer solutions (see Experimental Section).

The low values of K_d limit the enzyme concentration range over which determinations can be made. The enzyme concentration used in these experiments ranged between 0.2 μM and 2.0 μM. Over this range there was no noticeable effect of enzyme concentration on dissociation constant.

The binding of the coenzyme and its analogues appears to be on a strictly 1:1 stoichiometry. We have obtained no evidence for a second coenzyme binding site such as that described for the *E. coli* enzyme by Williams et al. (1973) and Poe et al. (1974).

Coenzyme Specificity of Dihydrofolate Reductase. Preliminary experiments on the relative rates of oxidation of the coenzyme analogues by dihydrofolate reductase have been performed. The reaction mixture contained approximately 0.04 μM enzyme, 50 μM DHF, 50 μM coenzyme, and 50 mM Tris, 500 mM KCl (pH 7.5) in a total volume of 3.1 mL. The oxidation rates were measured by following the change in extinction at the absorption maxima of the coenzyme analogues (P-L Biochemicals Inc., 1973).

The relative rates of oxidation of NADPH, deamino-NADPH, etheno-NADPH, and 3-acetylpyridine-NADPH were approximately 1:1:0.25:0.1, respectively. Under these conditions no oxidation of thionicotinamide-NADPH could be detected.

Qualitative Description of the Kinetics of Coenzyme Binding to Dihydrofolate Reductase. When NADPH or its structural analogues deamino-NADPH and etheno-NADPH were reacted in greater than equimolar amounts with the enzyme, the reaction curve, as monitored by dihydrofolate reductase fluorescence, showed a fast quench whose rate increased linearly with increasing ligand concentration, and a much slower, apparently first-order quench of rate approximately 0.03 s^{-1}. Under pseudo-first-order conditions no deviation from a single exponential could be detected for either phase. This behavior is consistent with a reaction scheme which

SCHEME I

$$E_1 + L \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E_1L$$

$$k_2 \uparrow\downarrow k_{-2}$$

$$E_1L^*$$

SCHEME II

$$E_1 + L \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E_1L$$

$$k_2 \uparrow\downarrow k_{-2}$$

$$E_2$$

involves a fast bimolecular ligand–protein association and a slow unimolecular protein isomerization, most probably a conformational transition. Two minimal kinetic schemes that conform with this behavior are as shown in Schemes I and II. In Scheme I it is the enzyme–ligand complex which isomerizes, whereas in Scheme II it is the free protein.

In a series of experiments in which the concentration of dihydrofolate reductase was kept constant and the relative concentration of coenzyme was varied, it was found that the amplitude of the fast phase when expressed as a percentage of the total signal change was not independent of the relative concentrations but rather increased toward 100% when less than stoichiometric amounts of ligand were reacted. Figure 2 shows representative results of such experiments performed at pH 6.0. Clearly these results are consistent with Scheme II but are inconsistent with a mechanism where the slow quench observed represents the isomerization of the binary complex and therefore Scheme I can be ruled out.

Preincubation of dihydrofolate reductase with varying subequimolar concentrations of NADPH followed by reaction with excess NADPH did not affect the relative amplitudes of the fast and slow phases nor the apparent rates of reaction. Furthermore, no effect of preincubation time between 2 min and 1 h was observed. Such observations are consistent with Scheme II in which there are two interconvertible forms of enzyme, E_1 and E_2. When excess NADPH is added, the fraction of enzyme in the E_1 form rapidly binds ligand in a bimolecular process which is virtually irreversible at working concentrations ($\sim 10^{-6}$ M) as a consequence of the very low dissociation constant of the binary complex (Table I). According to this scheme, the slow quench corresponds to the conversion of E_2 to E_1 followed by rapid NADPH binding.

The kinetics of binding of 3-acetylpyridine-NADPH, thionicotinamide-NADPH, and $NADP^+$ to dihydrofolate reductase are in many respects similar to those of NADPH, deamino-NADPH, and etheno-NADPH binding. However, in their case there appears to be one important difference in that the fluorescence quench of the "slow" phase does not fit well to a single exponential but can be resolved into two components of which one, the "intermediate phase", has a rate of approximately 0.5 s^{-1} and the other, the "slow phase", has a similar rate to that observed during NADPH binding. Although the percentage of the total signal change occurring in the fast phase appears to be independent of which coenzyme analogue is used, this is not the case for the intermediate and slow phases whose approximate relative amplitudes (fluorescence quench of intermediate phase/fluorescence quench of slow phase) at pH 6.0 are 0.36, 0.43, and 0.13 for $NADP^+$, thionicotinamide-NADPH, and 3-acetylpyridine-NADPH, respectively. Scheme II, proposed for NADPH binding, cannot, in its simplest form, account for the observation of this intermediate phase and thus any proposed scheme for analogue

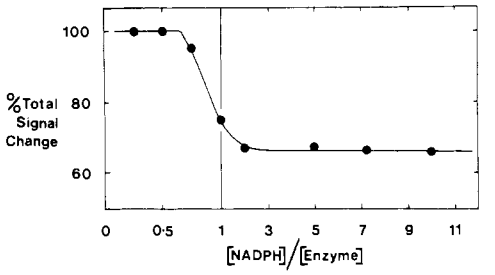


FIGURE 2: Effect of NADPH concentration on the percentage of the total quench occurring in the fast phase. Experiments performed at pH 6.0, 25 °C.

TABLE II: Association and Dissociation Rate Constants for Fast Phase Binding of Coenzymes to Dihydrofolate Reductase.[a]

Coenzyme	k_1 (M^{-1} s^{-1})	k_{-1} (s^{-1})
NADPH	1.6×10^7	<1
Deamino-NADPH	0.7×10^7	<1
Etheno-NADPH	1.6×10^7	<1
3-Acetylpyridine-NADPH	1.3×10^7	<1
Thionicotinamide-NADPH	1.5×10^7	41.5
$NADP^+$	0.8×10^7	133.2

[a] Values determined from concentration variation studies, pH 6.0 and 25 °C.

binding must be rather more complex if it is to be consistent with all the available data.

Experimentally, while the fast phase can easily be separated from any slower process or processes by using different time courses, such resolution of intermediate and slow rates is not so readily achieved. Therefore, these two slower phases were analyzed by collecting 200 data points for each phase using an appropriate time scale. The two reaction traces were then combined, omitting from the longer time course the data collected with greater precision in the shorter time course. After baseline adjustment to ensure data alignment every second data point was used in analytical fitting to two exponentials.

Effect of Ligand Concentration on Coenzyme Binding to Dihydrofolate Reductase. When the enzyme concentration was kept constant at 1 μM (final cuvette concentration after mixing) and the ligand concentration was varied, the observed rate of the fast phase increased linearly with increasing ligand concentration. For a simple association

$$E_1 + L \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E_1L$$

the observed rate (k_{app}) under pseudo-first-order conditions may be approximated by $k_{app} = k_1[L] + k_{-1}$, where k_1 and k_{-1} are the rate constants for the association and dissociation, respectively, and [L] is the ligand concentration. Thus a plot of k_{app} against [L] results in a straight line with slope k_1 and intercept k_{-1}. Assuming that this simple mechanism operates for the fast phase binding, these constants have been evaluated for each analogue at pH 6.0 and 25 °C and are shown in Table II.

Examination of Table II shows that for thionicotinamide-NADPH and $NADP^+$ the dissociation rates are quite substantial, indicating that the structure of the nicotinamide ring has a major effect on the lifetime of the complex and thus on the interatomic forces available in the complex between the ligand and the coenzyme binding site. For these analogues the dissociation constants calculated from the ratio of individual rate constants ($K_d = k_{-1}/k_1$) of 2.8×10^{-6} M for thionicoti-

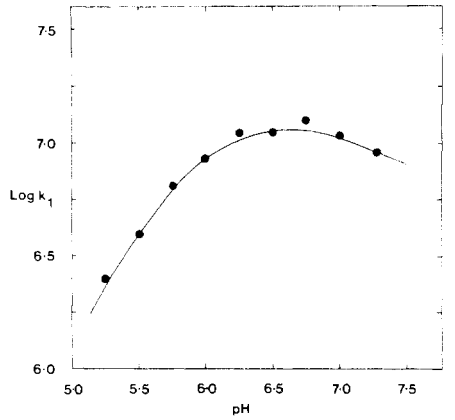


FIGURE 3: Effect of pH on the rate constant for fast phase binding of deamino-NADPH to the enzyme. Experiments performed at 25 °C.

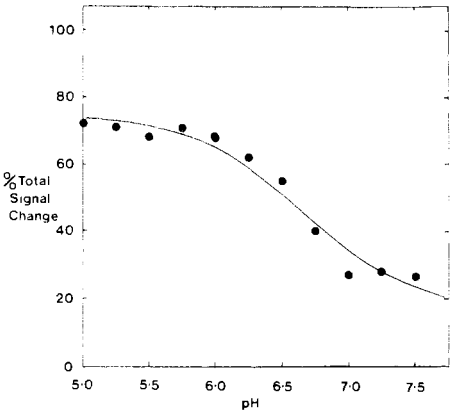


FIGURE 5: Effect of pH on the amplitude of the fast phase of NADPH binding to the enzyme. Experiments performed at 25 °C. Solid line is best fit to a single pK (6.6 ± 0.3).

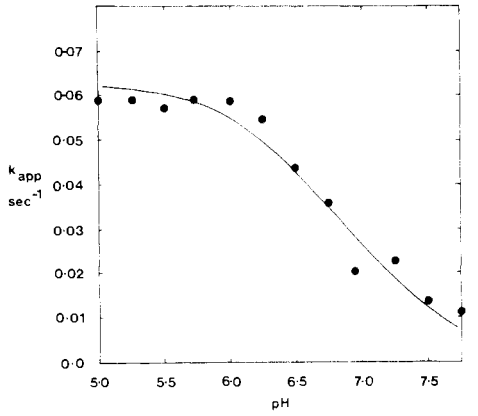


FIGURE 4: Effect of pH on the apparent rate of slow phase binding of NADPH to the enzyme. Experiments performed at 25 °C. Solid line is best fit to a single pK (6.8 ± 0.2).

TABLE III: Rates of Intermediate and Slow Phases of Coenzyme Binding to Dihydrofolate Reductase.[a]

Coenzyme	Intermediate phase k_{app} (s^{-1})	Slow phase k_{app} (s^{-1})
NADPH		0.056
Deamino-NADPH		0.044
Etheno-NADPH		0.038
3-Acetylpyridine-NADPH	0.87	0.039
Thionicotinamide-NADPH	0.71	0.039
$NADP^+$	0.35	0.035

[a] Reaction traces analyzed as two exponential decays, pH 6.0 and 25 °C.

namide-NADPH and 1.7×10^{-5} M for $NADP^+$ are in close agreement with those obtained from equilibrium titration experiments (Table I).

For all coenzymes examined variation of ligand concentration between 1 μM and 40 μM had little effect on the observed rate of the slow phase and, where present, of the intermediate phase. The only concentration effect detected was an increase (approximately 50%) in the rate of the slow phase when either NADPH, deamino-NADPH, or etheno-NADPH was reacted in less than stoichiometric amounts with the enzyme, under which condition the amplitude of the slow phase was very much reduced. A summary of the mean rates of each phase for each analogue at pH 6.0 is given in Table III.

Effect of pH on Coenzyme Binding to Dihydrofolate Reductase. The effect of pH on the kinetics of coenzyme binding to dihydrofolate reductase has been examined over the pH range 5.25–7.5. The well-documented instability of reduced pyridine coenzymes in acid solution (Kaplan, 1960) restricted experiments at lower pH values and in all cases conditions were chosen so that no detectable decomposition of ligand occurred during the course of the experiment.

The effect of pH on the rate of the fast phase showed some ligand dependence. With deamino-NADPH and etheno-NADPH this effect was most marked and is illustrated for deamino-NADPH in Figure 3. For 3-acetylpyridine-NADPH binding a qualitatively similar, though reduced, pH effect was apparent but the fast phase of NADPH binding showed no obvious pH dependence.

For all coenzymes the rates of both intermediate and slow phases were found to decrease in a similar manner with increasing pH. Figure 4 shows the pH dependence of the slow phase rate of NADPH binding to dihydrofolate reductase. In this figure the solid line is the best fit line to a single pK. A summary of the results obtained from this and similar experiments is given in Table IV. pH also had a marked effect on the relative amplitudes of each phase, although the total fluorescence signal change was relatively constant over the range examined. When excess coenzyme was reacted with dihydrofolate reductase, it was found that the amplitude of the fast phase decreased while the amplitude of the slow phase (and of the intermediate phase, where present) increased in a complementary manner with increasing pH. This effect on the amplitude of the fast phase expressed as a percentage of the total fluorescence quench when NADPH was reacted with the enzyme is shown in Figure 5. The best fit line for a single pK gave a value of 6.64 ± 0.26. However, there is no physical significance to a fit to a single pK since the limiting concentrations of enzyme species are not 0 and 100%. It is necessary to postulate the equilibrium system in Scheme III, in which all species can exist, and in which E_1H^+ and E_1 represent the tightly binding form. The values of K_4 and K_2 can be estimated from the plot of percentage fast phase against pH. K_1 and K_3 can then be calculated at each data point and mean values for each of the constants can be used as starting values for non-linear regression analysis of the data.

The values obtained for the pKs of the enzyme ionizations and the equilibrium constants of the two forms are summarized in Table V. The consistency of the values of the constants is encouraging in view of the relatively high experimental error

TABLE IV

Coenzyme	pH 5.25 k_{app} (s^{-1})	pH 7.5 k_{app} (s^{-1})	pK
(a) Effect of pH on the Rate of the Intermediate Phase of Coenzyme Binding to Dihydrofolate Reductase, 25 °C			
Thionicotinamide-NADPH	0.83	0.21	6.68 ± 0.48
$NADP^+$	0.55	0.15	6.37 ± 0.38
(b) Effect of pH on the Rate of the Slow Phase of Coenzyme Binding to Dihydrofolate Reductase, 25 °C			
NADPH	0.060	0.012	6.76 ± 0.18
Deamino-NADPH	0.058	0.009	6.40 ± 0.10
Etheno-NADPH	0.058	0.016	6.32 ± 0.05
3-Acetylpyridine-NADPH	0.043	0.015	6.46 ± 0.12
Thionicotinamide-NADPH	0.037	0.018	6.75 ± 0.18
$NADP^+$	0.040	0.018	7.03 ± 0.26

TABLE V: Equilibrium Constants and pK Values Determined from Analysis of Relative Amplitudes of Fast and Slow Phases at pH 6 and 25 °C.

Ligand	K_2	K_4	pK_1	pK_3
NADPH[a]	7.1 ± 4.7	0.4 ± 0.1	7.2 ± 0.3	6.0 ± 0.3
NADPH[b]	6.0 ± 2.9	0.3 ± 0.1	7.4 ± 0.2	6.2 ± 0.2
Deamino-NADPH	9.2 ± 4.0	0.3 ± 0.1	7.5 ± 0.3	6.0 ± 0.3
Etheno-NADPH	5.7 ± 1.7	0.3 ± 0.1	7.3 ± 0.2	6.0 ± 0.2

[a] Data from protein fluorescence quenching measurements. [b] Data from NADPH fluorescence enhancement measurements.

TABLE VI: Thermodynamic Parameters Determined for Reaction of Coenzyme and Analogues to Dihydrofolate Reductase, pH 6.0.

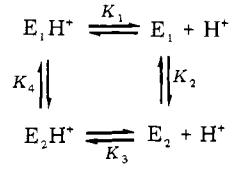
Ligand	E_a (kcal mol^{-1})	$\Delta H^{\ddagger}$ (kcal mol^{-1})	$\Delta S^{\ddagger}$ (cal mol^{-1} deg^{-1})	$\Delta G^{\ddagger}$ (kcal mol^{-1})
(a) Fast phase				
NADPH	2.5	1.9	−19.4	7.7
Deamino-NADPH	5.7	5.1	−10.7	8.3
Etheno-NADPH	0.9	0.3	−25.0	7.8
3-Acetylpyridine-NADPH	7.4	6.8	−2.7	7.6
(b) Intermediate phase				
3-Acetylpyridine-NADPH	14.0	13.4	−14.4	17.7
Thionicotinamide-NADPH	16.8	16.2	−5.0	17.7
$NADP^+$	15.6	15.0	−8.4	17.5
(c) Slow phase				
NADPH	12.6	12.0	−23.6	19.0
Deamino-NADPH	16.4	15.8	−11.4	19.2
Etheno-NADPH	11.8	11.2	−27.3	19.3
3-Acetylpyridine-NADPH	13.8	13.2	−20.8	19.4
Thionicotinamide-NADPH	15.5	14.9	−14.9	19.3
$NADP^+$	14.6	14.0	−17.9	19.3

associated with their determination. The similarity of the pH dependence of the slow phase rate for each coenzyme species is also consistent with the idea of a pH-dependent protein isomerization. At low pH the fraction of the enzyme in the E_1 (NADPH binding) form is increased which results in a corresponding increase in the amplitude of the fast phase.

Attempts to measure directly the interconversion rates of the enzymic species by pH-jump experiments have met with limited success. Using bromothymol blue as indicator it was found that the indicator bound to the enzyme and inhibited its activity. *p*-Nitrophenol did not bind to the enzyme nor inhibit its activity, but instrumental problems and difficulties associated with measuring the true final pH of the unbuffered enzyme solution have meant that all that can be said about these rates is that they are less than 0.1 s^{-1} and are therefore likely to be the slower of the two slow phases of the binding reaction.

Effects of Temperature and Viscosity on the Kinetics of Coenzyme Binding to Dihydrofolate Reductase. The effect of temperature on the kinetics of the binding of NADPH and analogues to dihydrofolate reductase has been investigated over the temperature range 11 to 37 °C at pH 6.0. Kinetic constants were determined under pseudo-first-order conditions in which ligand was reacted in at least tenfold molar excess over the enzyme. Under these conditions and when the dissociation rate, k_{-1}, is negligible as for NADPH, deamino-NADPH, etheno-NADPH, and 3-acetylpyridine-NADPH (Table II), k_1 of the fast phase could be estimated from experiments at a single ligand concentration from the relationship $k_{app} = k_1[L]$.

SCHEME III



In each experiment the data conformed to the Arrhenius law enabling the activation energy, E_a, to be calculated from the slope of a plot of ln k_{app} against the reciprocal of the absolute temperature. The thermodynamic parameters $\Delta H^{\ddagger}$ (enthalpy), $\Delta S^{\ddagger}$ (entropy), and $\Delta G^{\ddagger}$ (free energy) could then be calculated from the equations:

$$\Delta H^{\ddagger} = E_a - RT \quad (1)$$

$$\ln k = \ln\left(\frac{k'T}{\hbar} e^{\Delta S^{\ddagger}/R} e^{-\Delta H^{\ddagger}/RT}\right) \quad (2)$$

$$\Delta G^{\ddagger} = \Delta H^{\ddagger} - T\Delta S^{\ddagger} \quad (3)$$

where k is the rate constant, k' is the Boltzmann constant, and $\hbar$ is Planck's constant.

The results of these experiments for the fast, intermediate and slow phases are given in Table VI. In this table, the figures quoted for $\Delta H^{\ddagger}$, $\Delta S^{\ddagger}$, and $\Delta G^{\ddagger}$ are those for reaction at 25 °C (298 K).

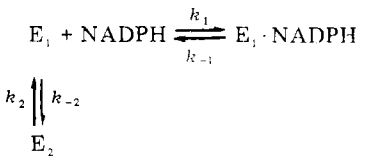
Structural modification of the coenzyme has been shown to have only a minor effect on the association rate constant (Table II). This, combined with the relatively high values for the rate constant (approximately 1.5×10^7 M^{-1} s^{-1}) and the

TABLE VII: Dissociation Rates of Coenzymes from Binary Complexes at pH 6.0 and 25 °C.[a]

Ligand	k_{-1} (s^{-1})	Competing ligand	Emission wavelength (nm)
NADPH	0.27	3-Acetylpyridine-NADPH	449
Deamino NADPH	0.19	3-Acetylpyridine-NADPH	449
3-Acetylpyridine-NADPH	3.1	NADPH	449
Thionicotinamide-NADPH	13.9	NADPH	341
$NADP^+$	40.3	NADPH	341
$NADP^+$	46.1	3-Acetylpyridine-NADPH	449

[a] Values determined from competition experiments using a coenzyme of different fluorescence quenching or energy transfer efficiency.

SCHEME IV



low activation energy of the fast phase of NADPH binding, suggest that the rate of formation of the binary complex may be diffusion limited. One of the characteristics of a diffusion-controlled reaction is that its rate is inversely proportional to the medium viscosity (Barnett, 1973). Therefore the binding of NADPH to dihydrofolate reductase was investigated using buffers containing 10–50% glycerol since water and glycerol have previously been shown to form relatively ideal mixtures (Stokes & Robinson, 1966; Schalager & Richards, 1970). The rate of the slow phase was, as expected, independent of the viscosity, whereas the rate of the fast phase increased linearly with the reciprocal of the percentage of glycerol in the medium.

Dissociation Rates from Competition Experiments. When a solution of enzyme-ligand complex (EL_1) is mixed with a solution containing a high concentration of some other compound, L_2, which competes for the ligand binding site on the enzyme, the following reactions take place

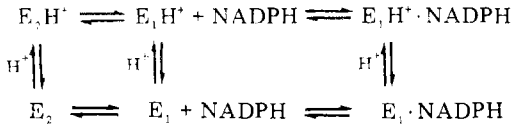
$$EL_1 \underset{k_1}{\overset{k_{-1}}{\rightleftharpoons}} E + L_1$$

$$E + L_2 \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EL_2$$

If $k_{-1} \ll k_2[L_2] \gg k_1[L_1]$, the signal due to the disappearance of EL_1 can be interpreted in terms of the rate constant, k_{-1}, for the dissociation of the complex. Under these conditions it has been possible to determine the dissociation rates for a number of dihydrofolate reductase-coenzyme complexes. In these experiments both the differences in efficiencies of fluorescence quenching and intensification of energy-transfer fluorescence of the various coenzyme analogues were employed. All reaction curves fitted well to a single exponential and the results are summarized in Table VII. In all experiments the excitation wavelength was 290 nm. Enzyme concentration was 1 μM and the concentrations of both bound and competing ligands were chosen so that the observed rate should be an accurate reflection of k_{-1}.

The experimental rate, k_{-1}, for the dissociation of $NADP^+$ from the binary complex given in Table VII is in agreement with the rate of $55 \pm 10\ s^{-1}$ estimated from NMR experiments (Way et al., 1975). However, the estimated rates of $NADP^+$ and thionicotinamide-NADPH dissociation given in this table differ by a factor of three from those given in Table II. One possible explanation for these results is that the bimolecular association of enzyme and ligand is followed by at least one transition of the binary complex which is not detected by fluorescence quenching or enhancement experiments, and that this reaction becomes rate limiting in the dissociation experiments.

SCHEME V



Discussion

The maximum value of 1.0×10^{-8} M found for the dissociation constant of NADPH from the *L. casei* MTX/R enzyme compares with values of 5×10^{-8} M for the L1210 lymphoma (Perkins & Bertino, 1966), 7.7×10^{-7} M for T_4 phage, and 9.1×10^{-8} for *E. coli* (Erickson & Matthews, 1973) and a value approximated as less than 10^{-8} M for this *L. casei* enzyme (Dann et al., 1976). However, since these other authors do not report the effect of pH on the value of K_d, a proper interspecies comparison cannot be made.

A comparison of the equilibrium dissociation constants for the complexes formed between dihydrofolate reductase and NADPH or coenzyme analogues indicates that the nicotinamide ring has a major influence in the binding process (Table I). Variation in these dissociation constants can be attributed largely to variation in the lifetimes of the complexes, since while the dissociation rates were ligand dependent, structural differences had little effect on the observed rates of complex formation (Tables II and VII).

The appearance of the biphasic reaction curve when dihydrofolate reductase was reacted with greater than stoichiometric concentrations of NADPH is consistent with a reaction scheme that includes a fast bimolecular NADPH-enzyme association and a much slower monomolecular protein transition. A detailed investigation of the concentration dependencies of both the rates and amplitudes of these phases has led to the proposal of Scheme IV for NADPH binding. According to this scheme the enzyme exists in two interconvertible forms, E_1 and E_2, to one of which, E_1, NADPH binds exclusively. Such a scheme adequately explains the biphasic decrease in protein fluorescence accompanying ligand binding since the rapid quench corresponding to the binding to E_1 is followed by a slow quench corresponding to the conversion of E_2 to E_1 followed by rapid coenzyme binding.

In the studies of pH dependencies of the slow phase rate (Figure 3) and relative amplitudes of the fast and slow phases (Figure 4), it has been shown that the percentage of the total enzyme in the E_1 form is pH dependent over the pH range 5.25 to 7.5. At low pH, the E_1 form, to which NADPH binds, predominates thus accounting for the increased fast phase amplitude. Scheme V for NADPH binding incorporates these pH effects. While this scheme adequately accounts for the kinetic data available for the binding of NADPH, deamino-NADPH, and etheno-NADPH, it cannot explain the third phase of in-

SCHEME VI

$$\begin{array}{ccc} E_1 + L & \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} & E_1L \\ k_2 \Big\| k_{-2} & & k_4 \Big\| k_{-4} \\ E_2 + L & \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} & E_2L \end{array}$$

termediate rate which is observed when 3-acetylpyridine-NADPH, thionicotinamide-NADPH, and $NADP^+$ bind to the enzyme. However, the percentage of the total quench occurring in the fast phase and the rate and thermodynamics of the slowest phase are fairly consistent for all the analogues examined. This suggests that the specific model proposed for NADPH binding may be generalized to account for the observed binding kinetics of the analogues by the inclusion of another unimolecular transition. As the amplitude of the intermediate phase increases with pH in a qualitatively similar manner to that of the slow phase, it is probable that, if such a transition exists, it is associated with the E_2 form of the enzyme which predominates at high pH. Therefore a possible model for analogue binding is as shown in Scheme VI (excluding pH effects). According to this model these analogues can bind to both enzymic forms, unlike the exclusive binding to E_1 of NADPH, deamino-NADPH, and etheno-NADPH, and the intermediate rate observed corresponds to the interconversion of the complexes, E_2L and E_1L. If this scheme is correct the affinities of E_1 and E_2 for ligand must be appreciably different for, if they were identical, all ligands would rapidly be taken up into complexed form and no slow phase, reflecting the interconversion of E_2 and E_1, would be observed. Furthermore, if the affinity constant for the binding of ligand to E_2 were low compared with the binding to E_1 and was due to a short lifetime (i.e., a high k_{-3}), the rate of this association step, which would be approximated by $k = k_3[L] + k_{-3}$, would be so fast and of such small amplitude that any fluorescence quench would be lost in the dead time of the instrument, thus explaining why a second fast association step is not observed. While this non-exclusive binding scheme appears to fit the experimental results in a qualitative manner, it is hoped that a more quantitative interpretation may come from a computer simulation of possible kinetic mechanisms.

The rate of the fast phase, corresponding to complex formation between ligand and enzyme, was largely unaffected by structural modification of the coenzyme (Table II). For a bimolecular reaction of a low molecular weight substrate with an enzymatic site Alberty & Hammes (1958) have calculated that the diffusion-limited rate constant should fall in the range of about 10^8 to 10^{10} M^{-1} s^{-1}. However, these calculations, which are based on the radial diffusion of a small ligand into a hemispherical site on the surface of the protein, would require that the ligand could encounter the binding site in any orientation. For a complex molecule such as NADPH (molecular weight 740) combining with a small enzyme such as dihydrofolate reductase (molecular weight 17 900) lower diffusion-limited rates would be expected. The association rates given in Table II are thus quite possibly consistent with the binding of coenzyme to dihydrofolate reductase being diffusion controlled. This interpretation has been further supported by the inverse dependence of the rate of NADPH-dihydrofolate reductase association on viscosity and by the low enthalpy of association which is close to that expected of diffusion in water (approximately 4 kcal mol^{-1}).

However, investigation of the pH dependence of the fast phase of deamino-NADPH binding has suggested that, after the bimolecular association, further transitions of the binary complex occur. Furthermore, NMR studies have shown that in the binary complex, the 2′-phosphate group of both NADPH and $NADP^+$ is in the dianionic state and its pK is shifted by at least 3 pH units from its value in the free coenzymes (Feeney et al., 1977). Therefore, at least one pH-dependent step must occur after complex formation. One scheme which may be considered is:

$$E_1 + L \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E_1L^* \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E_1L$$

where the conversion of E_1L^* to E_1L is pH dependent. Since, under pseudo-first-order conditions neither deviation of the fast phase rate from a single exponential rate constant nor dependence of the apparent rate constant on ligand concentration has been observed it may be assumed that E_1L^*, if it exists, is not significantly populated (King & Burgen, 1976). In such a situation the observed rate constant, k_{app}, may be approximated by:

$$k_{app} = \frac{k_1 k_2}{k_{-1} + k_2}$$

According to this relationship if k_2 is pH dependent, then the observed rate constant will also be pH dependent, which is apparently the case for the deamino-NADPH binding. Furthermore, if several rate constants contribute to the observed fast phase rate, this may also explain the enthalpies of association of deamino-NADPH and 3-acetylpyridine-NADPH which are higher than those expected from a diffusion-controlled reaction. The lack of pH-dependence of the experimental rate constant for NADPH binding to the enzyme can also be explained by applying the limiting condition that when $k_2 \gg k_{-1}$ the above expression reduces to $k_{app} = k_1$ which is the diffusion-limited association rate.

Acknowledgment

We would like to thank Dr. P. Campbell of NIBSC for his help with vialing of freeze-dried enzyme samples. We would also like to thank our colleagues in the Division of Molecular Pharmacology for helpful discussion.

References

Alberty, R. A., & Hammes, G. G. (1958) *J. Phys. Chem. 62,* 154–159.

Anson, M., & Bayley, P. (1976) *Rev. Sci. Instrum. 47,* 370–373.

Barnett, R. E. (1973) *Acc. Chem. Res. 6,* 41–46.

Blakley, R. L. (1969) *The Biochemistry of Folic Acid and Related Pteridines,* Elsevier, New York, N.Y.

Blakley, R. L., Schrock, M., Sommer, K., & Nixon, P. F. (1971) *Ann. N.Y. Acad. Sci. 186,* 119–130.

Dann, J. G. (1975) Ph.D. Thesis, Cambridge University.

Dann, J. G., Ostler, G., Bjur, R. A., King, R. W., Scudder, P., Turner, P. C., Roberts, G. C. K., Burgen, A. S. V., & Harding, N. G. L. (1976) *Biochem. J. 157,* 559–571.

Erickson, J. S., & Matthews, C. K. (1973) *Biochemistry 12,* 372–380.

Feeney, J., Birdsall, B., Roberts, G. C. K., & Burgen, A. S. V. (1977) manuscript in preparation.

Hanes, C. S., & Isherwood, F. A. (1949) *Nature (London) 164,* 1107–1112.

Kaplan, N. O. (1960) *Enzymes, 2nd Ed. 3,* 105.

King, R. W., & Burgen, A. S. V. (1976) *Proc. R. Soc. London, Ser. B 193,* 107–125.

Marquardt, D. W. (1963) *J. Soc. Ind. Appl. Math. 11,* 431–441.

Neef, V. G., & Huennekens, F. M. (1976) *Biochemistry 15,*

4042-4047.
Perkins, J. P., & Bertino, J. R. (1966) *Biochemistry 5*, 1005-1012.
P-L Biochemicals Inc. (1973) *UV Absorption Spectra of Pyridine Nucleotide Coenzymes and Coenzyme Analogs*, Milwaukee, Wis.
Poe, M., Greenfield, N. J., & Williams, M. N. (1974) *J. Biol. Chem. 249*, 2710-2716.
Roberts, G. C. K., Feeney, J., Burgen, A. S. V., Yuferov, V., Dann, J. G., & Bjur, R. A. (1974) *Biochemistry 13*, 5351-5357.
Schalager, L. L., & Richards, C. N. (1970) *J. Am. Chem. Soc. 92*, 5565-5570.
Shifrin, S., & Kaplan, N. O. (1958) *Proc. Natl. Acad. Sci. U.S.A. 44*, 177-181.
Stokes, R. H., & Robinson, R. A. (1966) *J. Phys. Chem. 70*, 2126-2130.
Udenfriend, S. (1962) *Fluorescence Assay in Biology and Medicine*, Academic Press, New York, N.Y.
Velick, S. F. (1958) *J. Biol. Chem. 233*, 1455-1467.
Wade, H. E., & Morgan, D. M. (1953) *Nature (London) 171*, 529-530.
Way, J. L., Birdsall, B., Feeney, J., Roberts, G. C. K., & Burgen, A. S. V. (1975) *Biochemistry 14*, 3470-3475.
Williams, M. N., Greenfield, N. J., & Hoogsteen, K. (1973) *J. Biol. Chem. 248*, 6380-6386.

Sites of Phosphorylation on Pyruvate Dehydrogenase from Bovine Kidney and Heart†

Stephen J. Yeaman, Eldridge T. Hutcheson,‡ Thomas E. Roche,§ Flora H. Pettit, James R. Brown, Lester J. Reed,* David C. Watson, and Gordon H. Dixon

ABSTRACT: The highly purified pyruvate dehydrogenase complex (EC 1.2.4.1) and uncomplexed pyruvate dehydrogenase from bovine kidney and heart mitochondria were phosphorylated and inactivated with pyruvate dehydrogenase kinase and [γ-^{32}P]ATP. Tryptic digestion of the phosphorylated pyruvate dehydrogenase yielded three phosphopeptides, a mono- (site 1) and a di- (sites 1 and 2) phosphorylated tetradecapeptide and a monophosphorylated nonapeptide (site 3). The amino acid sequences of the three phosphopeptides were established to be Tyr-His-Gly-His-Ser(P)-Met-Ser-Asn-Pro-Gly-Val-Ser-Tyr-Arg, Tyr-His-Gly-His-Ser(P)-Met-Ser-Asn-Pro-Gly-Val-Ser(P)-Tyr-Arg, and Tyr-Gly-Met-Gly-Thr-Ser(P)-Val-Glu-Arg. Phosphorylation proceeded markedly faster at site 1 than at sites 2 and 3, and phosphorylation at site 1 correlated closely with inactivation of pyruvate dehydrogenase. Complete inactivation of pyruvate dehydrogenase was associated with incorporation at site 1 of 1.0-1.6 mol of phosphoryl groups per mol of enzyme. Since pyruvate dehydrogenase is a tetramer ($\alpha_2\beta_2$) and since phosphorylation occurs only on the α subunit, the possibility of half-site reactivity is considered.

Activity of the mammalian pyruvate dehydrogenase complex is regulated by a phosphorylation-dephosphorylation cycle (Linn et al., 1969a,b). Phosphorylation and concomitant inactivation of the complex are catalyzed by a $MgATP^{2-}$-dependent kinase, and dephosphorylation and concomitant reactivation are catalyzed by a Mg^{2+}-dependent phosphatase. The site of this covalent regulation is the pyruvate dehydrogenase component of the complex. This component possesses the subunit composition $\alpha_2\beta_2$ (Barrera et al., 1972). Phosphorylation occurs on the α subunit (M_r 41 000). In this communication we present evidence that phosphorylation occurs on three serine residues (sites 1, 2, and 3) in the α subunit of bovine kidney and heart pyruvate dehydrogenase, and we report the amino acid sequences around the three phosphorylation sites. Evidence is also presented that inactivation of pyruvate dehydrogenase is associated with phosphorylation of the serine residue at site 1.

Experimental Procedure

Materials

The following materials were obtained from the sources cited: [γ-^{32}P]ATP (Amersham-Searle), L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone treated trypsin (Worthington); thermolysin, carboxypeptidase B, and aminopeptidase M (Sigma); carboxypeptidase Y (Pierce Chemicals); hexokinase (Boehringer-Mannheim). [γ-^{32}P]ATP was diluted at least 1:1000 with nonradioactive ATP (P-L Biochemicals). Thin-layer chromatography using ultraviolet light and radioautography for detection revealed the presence of only about 3% impurities. Highly purified preparations of the bovine kidney and heart pyruvate dehydrogenase complexes, the crystalline pyruvate dehydrogenase component, and bovine kidney PDH_a kinase were prepared as described previously (Linn et al., 1972). All other reagents and materials were of the purest grade available commercially.

† From the Clayton Foundation Biochemical Institute and the Department of Chemistry, The University of Texas at Austin, Austin, Texas 78712, and the Division of Medical Biochemistry, Faculty of Medicine, University of Calgary, Calgary, Alberta T2N 1N4, Canada (D.C.W. and G.H.D.). *Received January 18, 1978.* Supported in part by Grants GM06590 and HD02899 from the National Institutes of Health, United States Public Health Service.

‡ This work was abstracted in part from a dissertation submitted by E.T.H. to the Graduate School of The University of Texas at Austin in partial fulfillment of the requirements for the Ph.D. degree. Present address: Department of Biochemistry, University of Tennessee Center for Health Science, Memphis, Tennessee 38163.

§ Present address: Department of Biochemistry, Kansas State University, Manhattan, Kansas 66506.

0006-2960/78/0417-2364$01.00/0