Reisfeld, R. A., and Kahan, B. D. (1971), *Advan. Immunol. 12*, 117.
Schwartz, B. D., and Nathenson, S. G. (1971a), *J. Immunol. 107*, 1363.
Schwartz, B. D., and Nathenson, S. G. (1971b), *Transplant. Proc. 3*, 180.
Shapiro, A. L., Venuela, E., and Maizel, J. V., Jr. (1967), *Biochem. Biophys. Res. Commun. 28*, 815.
Shimada, A., and Nathenson, S. G. (1969), *Biochemistry 8*, 4048.
Shreffler, D. C., David, C. S., Passmore, H. C., and Klein, J. (1971), *Transplant. Proc. 3*, 176.
Snell, G. D. (1968), *in* Catalog of Mouse Alloantisera, Bethesda, Md., Transplantation Immunology Branch, National Institutes of Health, 1968, p 31.
Snell, G. D., Cherry, M., and Demant, P. (1971), *Transplant. Proc. 3*, 183.
Snell, G. D., and Stimpfling, J. H. (1966), *in* Biology of the Laboratory Mouse, Green, E., Ed., New York, N. Y., McGraw-Hill, p 706.

Equilibrium Kinetic Study of the Catalytic Mechanism of Bovine Liver Glutamate Dehydrogenase†

Emanuel Silverstein* and Guruprasad Sulebele‡

ABSTRACT: The catalytic mechanism of glutamate dehydrogenation with bovine liver glutamate dehydrogenase was studied by equilibrium kinetics utilizing α-[^{14}C]ketoglutarate to measure glutamate ⟷ α-ketoglutarate and [^{14}C]NAD(P)H to measure NAD(P)$^+$ ⟷ NAD(P)H rates in 0.1 M Tris-acetate at pH 8.0 and 25°. Increasing glutamate:α-ketoglutarate concentration resulted in increase in both glutamate ⟷ α-ketoglutarate and NAD$^+$ ⟷ NADH rates to plateau values while NADP$^+$ ⟷ NADPH rates were moderately depressed at elevated substrate levels (glutamate above about 0.04 M). Moderate depression of glutamate ⟷ α-ketoglutarate rates was obtained at elevated NAD$^+$:NADH concentration (NAD$^+$ greater than about 1 mM). The results are compatible with an alternative order of reactant addition and with enhancement of reactant binding by enzyme-bound reactant resulting in a decrease in the respective dissociation rates in ternary or quaternary complexes as compared to binary. Increasing concentrations of glutamate:NADH and α-ketoglutarate:NAD$^+$ resulted in marked and moderate inhibition, respectively, in both substrate and coenzyme interchanges, supporting the formation of the unreactive complex enzyme–NADH–glutamate and less facile formation of enzyme–NAD$^+$–α-ketoglutarate. Substrate interchange was usually considerably less than twice coenzyme interchange suggesting that chemical transformation may be of the same order of magnitude as the slower dissociation rates. NADP ⟷ NADPH rates were two–three times greater than glutamate ⟷ α-ketoglutarate rates below about 0.02 M glutamate, suggesting that under these conditions substrate rather than coenzyme dissociation may be rate limiting. Minimum estimates of dissociation constants from reciprocal plots of equilibrium rates and substrate concentrations were 66–360 μM (α-ketoglutarate), 1.8–4.5 μM (NADH), and 700–910 μM (NH_4^+). Simultaneous threefold depression of glutamate ⟷ α-ketoglutarate and NAD ⟷ NADH rates from maximum values at elevated glutamate:NH_4^+ concentrations suggested the possibility of simultaneous binding of both ligands in an unreactive or less reactive enzyme complex, resulting in decrease in the rate of chemical transformation.

Equilibrium kinetics studied by isotopic exchange is a powerful tool in the elucidation of enzyme mechanism (Boyer, 1959; Boyer and Silverstein, 1963; Silverstein 1963; Silverstein and Boyer, 1964a,b; Fromm *et al.*, 1964; Silverstein and Sulebele, 1969b,c). We have extended the application of equilibrium kinetics to the elucidation of the mechanism of enzyme modifier action and of the allosteric mechanism of regulatory enzymes which are of fundamental importance in metabolic control (Silverstein and Sulebele, 1966, 1967, 1969a,d, 1970a,b; Silverstein, 1970a–c). We have initially explored the application of equilibrium kinetics to a study of the mechanism of modifier action for the enzymes pig heart mitochondrial malate dehydrogenase (Silverstein and Sulebele, 1966, 1967, 1970b) and horse liver alcohol dehydrogenase (Silverstein and Sulebele, 1967; Silverstein, 1968, 1970a).

Subsequent equilibrium studies have been done with bovine liver glutamate dehydrogenase (L-glutamate:NADP$^+$ oxidoreductase (deaminating), EC 1.4.1.3) which is an important, well-studied allosteric enzyme at the interphase between amino acid and carbohydrate metabolism which catalyzes the reversible oxidative deamination of L-glutamate to α-ketoglutarate and ammonium ion (Frieden, 1963, 1964). The active oligomer has a mol wt of about 312,000 or 320,000, contains six apparently identical subunits arranged compactly in two identical triangular layers, and reversibly forms linear aggregates with molecular weights of up to several million (Eisenberg, 1970; Cassman and Schachman, 1971). The molecular weight per active center is 57,000 by spectrophotometric titration (Egan and Dalziel, 1971). Many studies have been made of the initial rate of catalytic activity and of the effect of various allosteric effectors on it and the state of ag-

† From the Laboratory of Molecular Biology, Department of Medicine, and the School of Graduate Studies, State University of New York, Downstate Medical Center, Brooklyn, New York 11203. *Received December 26, 1972.* This work was supported in part by grants from the National Science Foundation and the U. S. Public Health Service.

‡ Present address, Department of Chemical Technology, University of Bombay, Bombay-19, India.

gregation of oligomer (Frieden, 1963, 1964, 1968; Engel and Dalziel, 1969, 1970a,b; Markau *et al.*, 1971; Josephs and Borisy, 1972). The amino acid sequence of the monomer has been determined (Smith, *et al.*, 1970). Net initial rate kinetic studies have been interpreted as indicating either a compulsory order mechanism with differing orders (Frieden, 1959b; Corman *et al.*, 1967; Fahien and Strmecki, 1969) or a random order mechanism (Engel and Dalziel, 1970a,b).

We report here study by equilibrium kinetics of the catalytic mechanism of glutamate dehydrogenation by bovine liver glutamate dehydrogenase at pH 8.0 which indicates that an alternative order mechanism[1] (Boyer and Silverstein, 1963) exists. Equilibrium kinetic study of the allosteric mechanism of glutamate dehydrogenation and of the catalytic and allosteric mechanism of alanine dehydrogenation by bovine liver glutamate dehydrogenase, as well as studies on *Escherichia coli* asparate transcarbamylase, will be the subject of subsequent reports. Brief accounts of these studies have been given (Silverstein and Sulebele, 1969a,d, 1970a; Silverstein, 1970b,c)

Materials and Methods

Materials. Compounds used and their sources are: monosodium glutamic acid, α-ketoglutaric acid (sodium salt), disodium NADH, and DEAE-cellulose, Sigma Chemical Co., St. Louis, Mo.; ion-exchange purified NADH (lithium salt) and NAD^+ from P-L Biochemicals, Milwaukee, Wis.; $NADP^+$ and NADPH from Calbiochem, Los Angeles, Calif.; 2,4-dinitrophenylhydrazine from Eastman Kodak, Rochester, N. Y. [*carboxyl*-^{14}C]nicotinamide from Calbiochem (12 mCi/mmol) and from Amersham-Searle (60 mCi/mmol); α-[^{14}C]ketoglutaric acid (11 mCi/mmol) from Calbiochem; yeast glucose-6-phosphate dehydrogenase and bovine liver glutamate dehydrogenase (45 units/mg; three times recrystallized, dialyzed free of NH_4^+ against 10 mM sodium phosphate, pH 7.3, and made 50% in glycerol) from the Boehringer Mannheim Corp., New York, N. Y. The molecular weight of bovine liver glutamate dehydrogenase was taken as 3.2×10^5 (Cassman and Schachman, 1971).

Preparation of Radiosubstrates, Analysis of Purity, Substrate Separations. The preparation, storage, and analysis of purity of [*carboxyl*-^{14}C]NAD^+ have been described (Silverstein and Boyer, 1966; Silverstein, 1965).[2] [^{14}C]NADH was generated immediately prior to kinetic measurements as previously described (Silverstein and Boyer, 1964a) and absorbed from the 20-fold diluted reaction mixture after enzyme inactivation on a 0.7×2.5 cm DEAE-cellulose–acetate column. After elution of a small amount of unreacted [^{14}C]NAD^+ and nicotinamide with about 100 ml of 3.5 mM sodium acetate, [^{14}C]NADH was eluted with the buffer to be used for kinetic experiments (0.2 or 0.4 M Tris-acetate, pH 8.0). Separation of NAD^+ and NADH in reaction mixtures was accomplished by ion exchange chromatography on 1.8×3.2 cm DEAE-cellulose–HCO_3 columns; NAD^+ was eluted with 250 ml of 3.5 mM NH_4HCO_3 and NADH with 60 ml of 0.2 M NH_4HCO_3. Radioactivity of NADH was determined on a 3-ml aliquot in 15 ml of dioxane (17.5 g of 2,5-diphenyloxazole + 350 g of naphthalene per 3.5 l. of 1,4-dioxane).

[*carboxyl*-^{14}C]$NADP^+$ (1.5 or 2.5 mCi/mmol) was prepared by exchange reaction of $NADP^+$ with [*carboxyl*-^{14}C]nicotinamide catalyzed by bovine spleen nicotinamide adenine dinucleotidase, in a manner similar to that used for preparation of [^{14}C]NAD^+ (Silverstein and Boyer, 1966; Silverstein, 1965). [^{14}C]NADPH was enzymically generated from [^{14}C]$NADP^+$ immediately prior to kinetic measurements in a reaction mixture which consisted of 250 μM glucose 6-phosphate and 5 μg/ml of gucose-6-phosphate dehydrogenase in 2 ml of 5 mM sodium glycinate, pH 10.5. Enzyme was inactivated by addition of 250 μM $AgNO_3$ (final concentration); the mixture was diluted to 20–25 ml with water and absorbed on a 0.9×4 cm DEAE-cellulose–acetate column. Unreacted [^{14}C]$NADP^+$ and any [^{14}C]nicotinamide present were eluted with 200 ml of 0.05 M sodium acetate and [^{14}C]NADPH was eluted with 1.2 M Tris-acetate, pH 8.0. Fractions containing radioactivity were pooled and kept at 0° until use. [^{14}C]NADPH radioactivity enzymically convertible to [^{14}C]$NADP^+$ was determined by quantitative reaction of [^{14}C]NADPH in a 0.27-ml mixture containing 74 mM α-ketoglutarate, 74 mM ammonium acetate, and bovine liver glutamate dehydrogenase in 150 mM imidazole acetate at pH 7.0, followed by separation of $NADP^+$ and NADPH.

$NADP^+$ and NADPH were separated from reaction mixtures diluted 25-fold with water[2] on 1.8×3.2 cm DEAE-cellulose–HCO_3 columns (Silverstein, 1965). $NADP^+$ was eluted with 250 ml of 0.1 M NH_4HCO_3 and NADPH with 50 ml of 0.6 M NH_4HCO_3. The radioactivity of [^{14}C]NADPH was determined by scintillation counting of a 1.2-ml sample in 16 ml of scintillation fluid (7.0 ml of 0.5% 2,5-diphenyloxazole in toluene, 9 ml of 95% ethanol).

The purity of nonradioactive NADPH was determined by noting the change in absorbance at 340 nm after similar quantitative conversion of carefully weighed samples of NADPH with glutamate dehydrogenase (Horecker and Kornberg, 1948).

Enzymically reactive counts in α-[^{14}C]ketoglutarate were determined by quantitatively converting α-[^{14}C]ketoglutarate to [^{14}C]glutamate in a reaction mixture containing 148 mM imidazole acetate, pH 7.0, 0.3 μmol NADH, 20 μmol of ammonium acetate, and bovine liver glutamate dehydrogenase in 0.27 ml. α-Ketoglutarate was separated from glutamate by quantitative precipitation of α-ketoglutarate 2,4-dinitrophenylhydrazone by addition of 2 ml of 0.5% 2,4-dinitrophenylhydrazone, followed by 25–30 μmol of nonradioactive α-ketoglutaric acid and incubation for 20 min at 25° and 30 min at 0°. After addition of 3 drops of horse serum for ease of compaction of the precipitate and 0.2 ml of 1% Triton X-100, the α-ketoglutarate hydrazone was collected by centrifugation, washed with 3 ml of cold distilled water and 0.2 ml of 1% Triton X-100, drained dry, and dissolved in 1 ml of pyridine. The pyridine solution was plated and dried on an aluminum planchet fitted with lens tissue and the radioactivity determined by gas-flow counting in a low background Tracerlab omniguard counter. Storage of α-[^{14}C]ketoglutaric acid for 6 days at about 25, 4, or −15° in water, 0.2 M Tris-acetate (pH 8.0), water containing a drop of benzene, 0.1 N HCl, 1.0 N HCl, or 0.1 N NaOH resulted in maintenance of the original 97–98% radiopurity except for storage in water at 25° where a drop in radiopurity to 63% was observed. The decrease in radiopurity was due mainly to a decrease of total counts precipitable as the 2,4-dinitrophenylhydrazone, probably by decarboxylation of the labeled carboxyl.

[1] An *alternative order* mechanism is a branched sequence mechanism in which reactant ligands may bind to and dissociate from enzyme in any order. It includes a *random* mechanism in which the rate of ligand binding to and dissociation from enzyme is independent of other bound reactant ligands, and mechanisms in which the rate of reactant ligand binding and dissociation is not independent of other reactant ligands, such as a *partially compulsory pathway* (Boyer and Silverstein, 1963).

[2] Distilled and deionized water was used throughout except for occasional use of distilled water for dilution of coenzyme reaction mixtures after cessation of reaction.

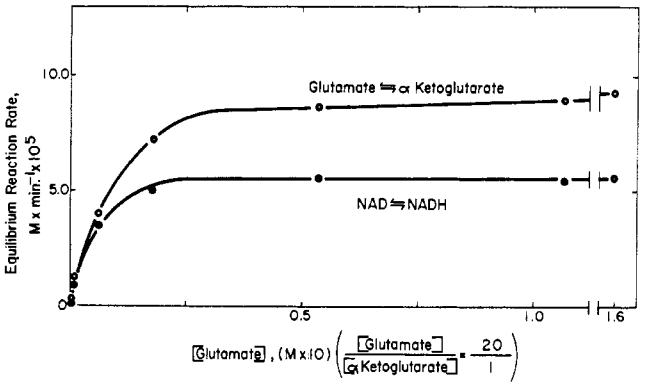

FIGURE 1: Effect of glutamate and α-ketoglutarate concentrations on the glutamate ⟷ α-ketoglutarate and NAD^+ ⟷ NADH reaction rates at equilibrium catalyzed by bovine liver glutamate dehydrogenase. The reaction mixture contained 4.05 mM NAD^+, 117.7 μM NADH, 5 mM ammonium acetate, 82.8 nM glutamate dehydrogenase, and glutamate and α-ketoglutarate as indicated in 0.25 ml of 106 mM Tris-acetate at pH 8.0 and 25°.

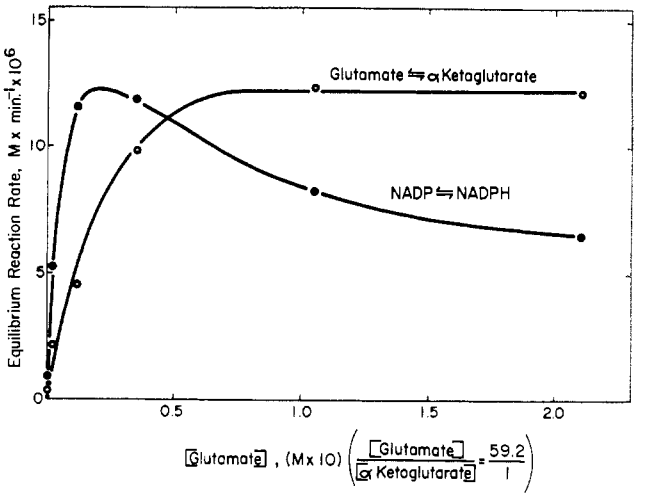

FIGURE 2: Effect of glutamate and α-ketoglutarate concentrations on the glutamate ⟷ α-ketoglutarate and $NADP^+$ ⟷ NADPH reaction rates at equilibrium catalyzed by bovine liver glutamate dehydrogenase. Reaction mixtures contained 3.98 mM $NADP^+$, 825 μM NADPH, 835 μM ammonium acetate, 166 nM glutamate dehydrogenase, and glutamate and α-ketoglutarate as indicated in 0.3 ml of 90 mM Tris-acetate at pH 8.0 and 25°.

Substrate concentrations and purity of the pyridine nucleotides were determined enzymically utilizing the absorbance of reduced pyridine nucleotide at 340 nm (Horecker and Kornberg, 1948). NAD^+ and NADH concentrations were determined with alcohol and lactate dehydrogenases (Silverstein, 1963; Silverstein and Sulebele, 1969b). $NADP^+$ concentration was determined by quantitative reaction to NADPH with glucose-6-phosphate dehydrogenase, and NADPH by quantitative reaction to $NADP^+$ utilizing the reaction mixtures already described for determination of convertibility to [^{14}C]-$NADP^+$ of enzymically generated [^{14}C]NADPH. Sodium glutamate revealed only a single ninhydrin spot on paper chromatography (*n*-butyl alcohol–glacial acetic acid–water, 100:22:50, v/v, 18 hr, descending on Whatman No. 1 paper.

The initial rate of α-ketoglutarate reduction was assayed in a reaction mixture containing 5.12 mM α-ketoglutarate, 125 μM NADH or NADPH, and 9.88 mM ammonium acetate in 98.8 mM Tris-acetate at pH 8.0 and 25°.

Reaction termination was achieved by enzyme inactivation by placing the reaction test tube at 100° for 25 sec followed by 0° and diluting 120-fold with water in the case of NAD^+ ⟷ NADH and $NADP^+$ ⟷ NADPH equilibrium rate measurements and by addition of a tenfold greater volume of 0.5% 2,4-dinitrophenylhydrazone in 4 N HCl in the case of α-ketoglutarate ⟷ glutamate equilibrium rate measurements. The two methods were shown to be identically effective by testing both with α-ketoglutarate ⟷ glutamate reactions. After 15–20 sec at 100° with or without an additional period at 0° the glutamate dehydrogenase in equilibrium reaction mixtures became totally inactive in catalyzing α-ketoglutarate reaction with NADH and NH_4^+.

Experimental Design for Kinetic Experiments at Equilibrium. Equilibrium mixtures were made of substrates prepared at pH 8.0 in water[2] immediately prior to each experiment and stored at 0° until use. Absorbance at 340 nm was taken at 25° before and after addition of glutamate dehydrogenase to ensure the presence of equilibrium and to quantitate and correct for any substrate shift. [^{14}C]NADH or [^{14}C]NADPH and α-[^{14}C]ketoglutarate, respectively, were added in triplicate to separate 0.2-ml aliquots of reaction mixtures at 25°. The reaction was stopped after attainment of about 50–80% of isotopic equilibrium. Unreacted radiosubstrate was determined in triplicate or quadruplicate by enzyme inactivation prior to addition of radiosubstrate. Reaction rates were calculated from initial and final radiosubstrate activity, reaction time, and equilibrium substrate concentration (Boyer, 1959).

Results

Direct Proportionality of Reaction with Time. The extent of the glutamate ⟷ α-ketoglutarate and NAD^+ ⟷ NADH reactions at equilibrium was linear with time. This relationship allowed for measurement of disparate rates by utilization of different reaction times.

Effect of Substrate Concentration on Reaction Rates at Equilibrium. Increasing glutamate:α-ketoglutarate[3] concentration from highly unsaturated to saturating levels resulted in increase in both glutamate ⟷ α-ketoglutarate and NAD^+ ⟷ NADH equilibrium rates to a plateau, ruling out a compulsory pathway in which coenzyme binds prior to substrate (Figure 1). The coenzyme interchange rate was more than half the substrate rate and was thus much closer to the substrate rate than was the case for lactate, alcohol, and malate dehydrogenases previously studied (Silverstein and Boyer, 1964a,b; Silverstein and Sulebele, 1969b,c). The initial rate of α-ketoglutarate reduction was 1.2–1.8 times the maximum NAD^+ ⟷ NADH rate and 0.95–1.1 times the maximum glutamate ⟷ α-ketoglutarate rate.

Increasing glutamate:α-ketoglutarate concentration at constant $NADP^+$ and NADPH concentration also resulted in a rise in the glutamate ⟷ α-ketoglutarate equilibrium reaction rate to a plateau value (Figure 2). The $NADP^+$ ⟷ NADPH equilibrium reaction rate, on the other hand, initially rose more steeply than the glutamate ⟷ α-ketoglutarate rate and gradually fell at glutamate concentrations above 40 mM to about half its maximum value. This result is compatible with an alternative order of substrate addition with $NADP^+$ as coenzyme and slower dissociation of coenzyme from ternary as compared to binary complexes, as has been found for several

[3] Glutamate:α-ketoglutarate concentration signifies glutamate and α-ketoglutarate concentration at constant ratio. This designation is used for other reactant concentrations as well.

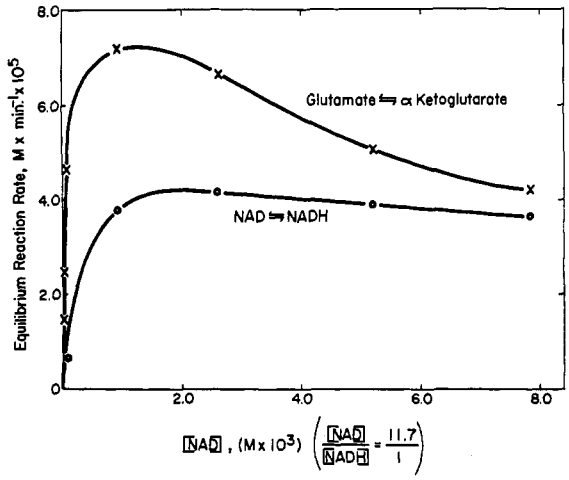

FIGURE 3: Effect of NAD^+ and NADH concentration on the glutamate ⇀↽ α-ketoglutarate and NAD^+ ⇀↽ NADH reaction rates at equilibrium catalyzed by bovine liver glutamate dehydrogenase. The reaction mixture contained 160 mM glutamate, 4 mM α-ketoglutarate, 5 mM ammonium acetate, 82.8 mM glutamate dehydrogenase, and NAD^+ and NADH as indicated in 0.25 ml of 106 mM Tris-acetate at pH 8.0 and 25°.

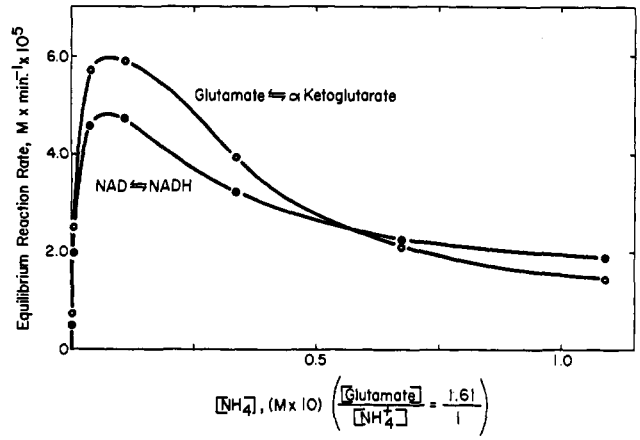

FIGURE 4: Effect of NH_4^+ and glutamate concentration on the glutamate ⇀↽ α-ketoglutarate and NAD^+ ⇀↽ NADH reaction rates at equilibrium catalyzed by bovine liver glutamate dehydrogenase. The reaction mixture contained 4 mM NAD^+, 126 μM NADH, 400 μM α-ketoglutarate, 82.8 nM glutamate dehydrogenase, and glutamate and ammonium acetate as indicated in 0.25 ml of 106 mM Tris-acetate, at pH 8.0 and 25°

other systems (Silverstein, 1963; Boyer and Silverstein, 1963; Silverstein and Boyer, 1964a,b; Silverstein and Sulebele, 1969b,c). Unlike the case with NAD^+ as coenzyme (Figure 1), the $NADP^+$ ⇀↽ NADPH equilibrium reaction rate was at least twofold higher than the glutamate ⇀↽ α-ketoglutarate equilibrium reaction rate up to a concentration of glutamate in excess of 10 mM, suggesting that substrate dissociation may be rate limiting under these conditions. The initial rate of α-ketoglutarate reduction with $NADP^+$ was 6.1 times the maximum glutamate ⇀↽ α-ketoglutarate and $NADP^+$ ⇀↽ NADPH equilibrium reaction rates. It is of interest that the initial rate of α-ketoglutarate reduction with NADPH was 2.5 or 3.8 times the rate with NADH, while the rate of oxidative deamination of glutamate with NAD^+ was 5.4 times that with $NADP^+$ under the experimental conditions used (Table I).

In Figure 3 increasing NAD^+:NADH concentration is shown to result in a rise of the NAD^+ ⇀↽ NADH equilibrium reaction rate to a plateau value, and in a sharp increase followed by a gradual fall in the glutamate ⇀↽ α-ketoglutarate rate to near equality with the NAD^+ ⇀↽ NADH rate. This finding rules out a compulsory pathway mechanism with substrate binding prior to coenzyme. The initial rate of α-ketoglutarate reduction was 1.4 times the maximum glutamate ⇀↽ α-ketoglutarate rate and 2.3 times the maximum NAD^+ ⇀↽ NADH rate.

When glutamate:NH_4^+ concentration was increasingly elevated, a sharp rise in both glutamate ⇀↽ α-ketoglutarate and NAD^+ ⇀↽ NADH equilibrium reaction rates was found, followed by a gradual, persistent fall, slightly greater for NAD^+ ⇀↽ NADH, suggesting a diminution in the rate of chemical transformation and possibly in the rate of dissociation (Figure 4). The initial rate of α-ketoglutarate reduction was 1.7 times the maximum glutamate ⇀↽ α-ketoglutarate equilibrium reaction rate and 2.0 times the maximum NAD^+ ⇀↽ NADH rate.

Increasing concentration of glutamate:NADH resulted in an increase in glutamate ⇀↽ α-ketoglutarate and NAD^+ ⇀↽ NADH equilibrium reaction rates followed by a decrease to about one-third of the maximum values (Figure 5). This finding is compatible with substantial formation of the abortive (unreactive) complex enzyme–glutamate–NADH (Silverstein, 1963; Boyer and Silverstein, 1963; Silverstein and Sulebele, 1969b,c). The decrease in the glutamate ⇀↽ α-ketoglutarate rate was somewhat greater than the NAD^+ ⇀↽ NADH rate, so that the glutamate ⇀↽ α-ketoglutarate rate was slightly greater than the NAD^+ ⇀↽ NADH rate at maximum values and slightly smaller after inhibition. The initial rate of α-ketoglutarate reduction was 11.4 times greater than the maximum glutamate ⇀↽ α-ketoglutarate equilibrium reaction rates and 12.9 times the maximum NAD^+ ⇀↽ NADH rate.

Similarly, a rise followed by a moderate fall in both glutamate ⇀↽ α-ketoglutarate and NAD^+ ⇀↽ NADH equilibrium reaction rates was found when the nonreactive pair α-ketoglutarate–NAD^+ was increased from below K_m values to above saturation (Figure 6), suggesting moderate formation of the abortive complex enzyme–NAD^+–α-ketoglutarate. The

TABLE I: Initial Net Reaction Rates of α-Ketoglutarate Reduction and Glutamate Oxidative Deamination Catalyzed by Bovine Liver Glutamate Dehydrogenase.

Coenzyme	Rate (M × min^{-1} × mg^{-1} × 10^5) α-Ketoglutarate → Glutamate	Glutamate → α-Ketoglutarate[c]
NAD(H)	187[a]	199
	186[b]	
NADP(H)	705[a]	36.9
	469[b]	

[a] Reaction mixtures of 2.0 ml contained 6.18 mM α-ketoglutarate, 125 μM NADH or NADPH, 0.1 M ammonium acetate, and 31.3 nM enzyme in 0.2 M Tris-acetate at pH 8.0 and 25°. [b] Reaction mixtures of 2.0 ml contained 5.09 mM α-ketoglutarate, 116 μM NADH or 114 μM NADPH, 9.88 mM ammonium acetate, and 31.3 nM enzyme in 0.1 M Tris-acetate at pH 8.0 and 25°. [c] Reaction mixtures of 2.0 ml contained 20 mM sodium glutamate, 960 μM NAD^+ or $NADP^+$, and 31.3 nM enzyme in 0.1 M Tris-acetate at pH 8.0 and 25°.

TABLE II: Estimates of Minimum Values for Dissociation Constants from $1/R'$ *vs.* $1/S$ and $1/R$ *vs.* $1/S$ Plots.[a]

Substrate	Condition	Dissociation Constant		
		from $1/R'$ *vs.* $1/S$	from $1/R$ *vs.* $1/S$	from net initial rate kinetics or spectroscopy
α-Ketoglutarate	Variable α-ketoglutarate:glutamate Constant NAD^+:NADH	3.6×10^{-4}	2.5×10^{-4}	7×10^{-4} [b] 5.6×10^{-4} [c] 28×10^{-4} [d]
	Variable α-ketoglutarate:glutamate Constant $NADP^+$:NADPH	1.7×10^{-4}	0.66×10^{-4}	
NADH	Variable NAD^+:NADH Constant $NADP^+$:NADPH	1.8×10^{-6}	4.5×10^{-6}	24×10^{-6} [c]
NH_4^+	Variable glutamate:NH_4^+ Constant NAD^+:NADH	9.1×10^{-4}	7.0×10^{-4}	32×10^{-4} [b] 790×10^{-4} [c]

[a] 0.1 M Tris-acetate, pH 8.0, 25°. R' refers to the glutamate ⇀↽ α-ketoglutarate and R to the NAD^+ ⇀↽ NADH reaction rates at equilibrium. [b] K_m; 10 mM Tris-acetate at pH 8.0 and 25° (Frieden, 1963). [c] Constants for dissociation of ligands from the enzyme–NADH–α-ketoglutarate–NH_4^+ complex, calculated from kinetic data; phosphate buffer, pH 7.0, $I = 0.25$, 25° (Engel and Dalziel, 1970b). [d] Dissociation constant from ultraviolet difference spectroscopy (Cross *et al.*, 1972).

initial rate of α-ketoglutarate reduction was 1.7 times the maximum glutamate ⇀↽ α-ketoglutarate equilibrium reaction rate and 2.4 times the maximum NAD^+ ⇀↽ NADH rate.

Estimates of minimum values of dissociation constants may be obtained from the ratio of slope to intercept of reciprocal plots of equilibrium reaction rate *vs.* substrate concentration (Boyer and Silverstein, 1963; Silverstein and Boyer, 1964a; Silverstein and Sulebele, 1969b,c). Estimates given for α-ketoglutarate, NADH, and NH_4^+ are generally lower than K_m or dissociation constants obtained from net initial reaction kinetics (Table II), suggesting that k' may be $>k$ since the dissociation constant estimate $= K/[1 + (k'/k)]$ (Figure 7; Boyer and Silverstein, 1963; k and k' are the chemical transformation rates for the forward and reverse reactions, respectively).

Discussion

The catalytic mechanism for bovine liver glutamate dehydrogenase suggested by these experiments may be termed alternative order[1] substrate addition in which substrates and coenzymes may bind and dissociate in any order (Figure 7) (Boyer and Silverstein, 1963; Silverstein, 1963). This conclusion is compatible with the results of recent initial net reaction

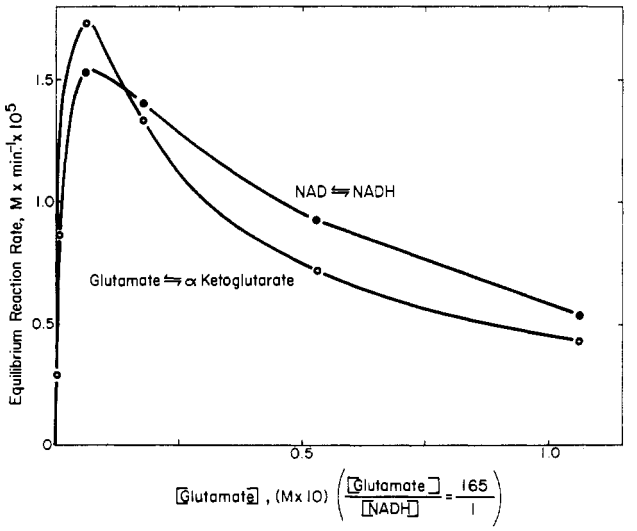

FIGURE 5: Effect of glutamate and NADH concentrations on the glutamate ⇀↽ α-ketoglutarate and NAD^+ ⇀↽ NADH reaction rates at equilibrium catalyzed by bovine liver glutamate dehydrogenase. The reaction mixtures contained 125 μM α-ketoglutarate, 373 μM NAD^+, 5 mM ammonium acetate, 166 nM glutamate dehydrogenase, and glutamate and NADH as indicated in 0.25 ml of 106 mM Tris-acetate at pH 8.0 and 25°.

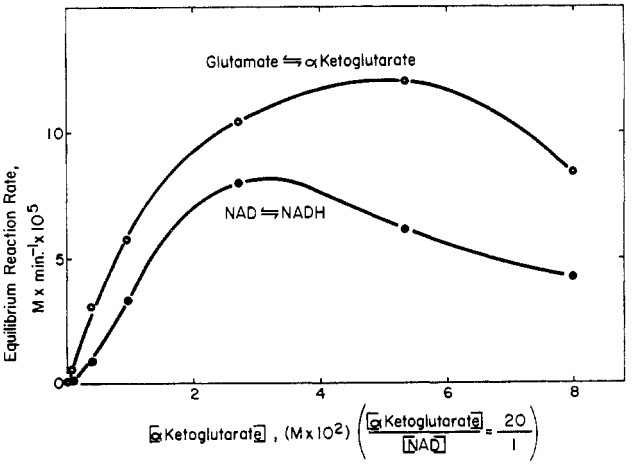

FIGURE 6: Effect of α-ketoglutarate and NAD^+ concentrations on glutamate ⇀↽ α-ketoglutarate and NAD^+ ⇀↽ NADH reaction rates at equilibrium catalyzed by bovine liver glutamate dehydrogenase. The reaction mixtures consisted of 160 mM glutamate, 500 μM ammonium acetate, 125 μM NADH, 166 nM glutamate dehydrogenase, and α-ketoglutarate and NAD^+ as indicated in 0.25 ml of 106 mM Tris-acetate at pH 8.0 and 25°.

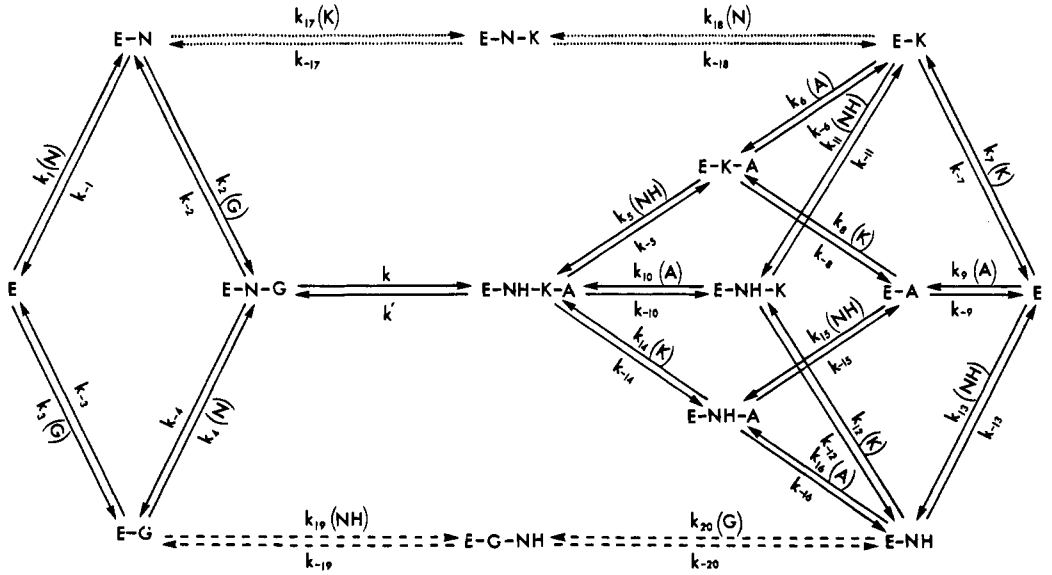

FIGURE 7: Alternative order kinetic scheme for the reaction: glutamate + $NAD(P)^+$ + H_2O ⇀ α-ketoglutarate + NH_4^+ + NAD(P)H + H^+. Chemical transformation occurs in catalytically effective and interconvertible ternary and quaternary complexes. Catalytically effective steps are indicated by solid arrows and catalytically ineffective steps by dashed (quantitatively significant) and dotted (quantitatively small) arrows leading to unreactive ternary complexes. Other more hypothetical complexes such as additional NH_4^+ containing complexes are not included: G, glutamate; K, α-ketoglutarate; N, $NAD(P)^+$; NH, NAD(P)H; A, NH_4^+.

kinetic studies (Engel and Dalziel, 1970a,b), with evidence by ultraviolet difference spectroscopy for binary complexes of glutamate dehydrogenase with α-ketoglutarate (Cross *et al.*, 1972), L-glutamate (Prough *et al.*, 1972a), and L-leucine (Prough *et al.*, 1972b), and with a recent transient kinetic study suggesting random binding of $NADP^+$ and glutamate (Colen *et al.*, 1972). Compulsory pathways or binding orders involving obligatory binding of substrate or coenzyme prior to coreactant are ruled out by these results. Thus, increasing glutamate:α-ketoglutarate or NAD^+:NADH concentration to saturating levels would have been expected to fully suppress the coenzyme or substrate equilibrium interchanges, respectively, if a compulsory pathway exists (Boyer, 1959; Silverstein, 1963). For example, if coenzyme obligatorily bound first to the enzyme, increasingly elevated glutamate:α-ketoglutarate concentration would effectively almost completely suppress coenzyme isotopic exchange since saturation of the substrate binding site would greatly suppress coenzyme dissociation and therefore also interchange of enzyme-bound coenzyme with the vastly greater pool of coenzyme in free solution. Thus, while coenzyme interchange could occur just as rapidly on the enzyme, isotopic exchange of the bulk of coenzyme which is in free solution would be prevented.

The reaction mechanism with $NADP^+$ as coenzyme instead of NAD^+, while also failing to exhibit an ordered reaction mechanism, differs in that moderate depression in the $NADP^+$ ⇀ NADPH rate occurs with elevated glutamate:α-ketoglutarate concentration (Figure 2). This finding is compatible with an alternative order mechanism with a partially compulsory order (Boyer and Silverstein, 1963) in which all reactants may freely bind and dissociate from the enzyme, but the rates of dissociation of $NADP^+$ and NADPH are faster from enzyme–coenzyme complexes than from enzyme–coenzyme–substrate complexes.[1]

An analogous situation obtains with respect to glutamate ⇀ α-ketoglutarate. The moderate inhibition of the glutamate ⇀ α-ketoglutarate equilibrium reaction rate to near equality with the NAD^+ ⇀ NADH rate at increasingly saturated NAD^+: NADH concentration is compatible with more rapid dissociation of glutamate and α-ketoglutarate from enzyme–substrate complexes lacking bound coenzyme. This rate effect may also be termed a partially compulsory reaction pathway. These experiments thus indicate that the binding of substrate and coenzyme has a mutually inhibitory effect on each other's dissociation. It is not clear whether the molecules may directly sterically hinder each other or whether an indirect effect on each other's dissociation could occur by perhaps subtle enzyme conformational change or change in the electronic configuration *per se* at the binding site. Spectral evidence for mutual enhancement of binding has recently been obtained for NADP(H) and α-ketoglutarate or glutamate (Cross, 1972; Cross *et al.*, 1972) and glutarate enhances the binding of NAD^+ and $NADP^+$ as determined by equilibrium dialysis (Dalziel and Egan, 1972).

Comparison of kinetics with NAD^+ and $NADP^+$ reveals an interesting difference. The $NADP^+$ ⇀ NADPH rate is severalfold higher than the glutamate ⇀ α-ketoglutarate equilibrium reaction rate below about 10 mM glutamate, and becomes smaller after about 50 mM glutamate. The NAD^+ ⇀ NADH rate on the other hand was always lower than the glutamate ⇀ α-ketoglutarate rate. These findings suggest that with NAD^+ as coenzyme, the coenzyme dissociation rates may be rate limiting for the overall reaction. On the other hand, the results with $NADP^+$ as coenzyme suggested that substrate dissociation is rate limiting below 50 mM glutamate under the conditions of these experiments, while coenzyme dissociation becomes rate limiting above 50 mM glutamate. In this regard it is of interest that in stopped-flow transient kinetic experiments of glutamate oxidation with $NADP^+$ and liver glutamate dehydrogenase which implicated NADPH dissociation as rate limiting, the glutamate concentration used was higher than 50 mM (Iwatsubo and Pantaloni, 1967; Fisher *et al.*, 1970; DiFranco and Iwatsubo, 1971). In view of the greater complexity of the equilibrium system here reported, as well as the results obtained, the precise relationship between the differences obtained with NAD(H) and NADP(H) and those previously re-

ported (possible noncatalytic center binding by NAD(H), disaggregation to oligomer by NADH, and significant inhibition by NADH and activation by NAD^+ (Frieden, 1959a; Pantaloni and Dessen, 1969; Jallon and Iwatsubo, 1971)) is not clear. Of the dehydrogenases previously studied by equilibrium kinetics (Silverstein and Boyer, 1964a,b; Silverstein and Sulebele, 1969b,c) bovine liver glutamate dehydrogenase is the only one to exhibit a lower equilibrium reaction rate for substrate than for coenzyme.

The lower equilibrium rates with $NADP^+$ as coenzyme as compared to NAD^+ (Figures 1 and 2) are correlated with the lower rate of glutamate oxidative deamination with $NADP^+$ as compared to NAD^+ (Table I), perhaps due to more rapid rate-limiting dissociation from enzyme (Iwatsubo and Pantaloni, 1967) of NADH than NADPH. Mutual enhancement of binding by ternary and quaternary complex ligands may also be greater with $NADP^+$ than with NAD^+, and various enzyme–substrate intermediates present at equilibrium may divert more of the enzyme from direct catalytic action with $NADP^+$ as coenzyme rather than NAD^+. The 20-fold greater rate of α-ketoglutarate reduction as compared to glutamate oxidation with NADP(H) suggests a remarkably increased binding of NADPH containing a reduced nicotinamide ring as compared to $NADP^+$. This marked difference in rate was not observed with NADH (Table I). The increase in the rate of α-ketoglutarate reduction with NADPH but not with NADH, associated with an increase of the NH_4^+ concentration from 9.9 to 100 mM, suggests tighter binding of NH_4^+ and therefore saturation at the lower NH_4^+ concentration in the presence of NADH but not with NADPH (Table I). The faster glutamate oxidation with NAD^+ and α-ketoglutarate reduction with NADPH (Table I), assuming rate-limiting coenzyme dissociation, suggests that addition of a phosphate group to the second carbon of the ribose of NAD^+ results in more rapid dissociation from enzyme of oxidized coenzyme and much less rapid dissociation of reduced coenzyme in reversible glutamate oxidative deamination with bovine liver glutamate dehydrogenase.

It should be noted, however, that stopped-flow transient kinetic studies which suggest rate limitation of NADPH dissociation in the oxidative deamination of glutamate with glutamate dehydrogenase at this time offer no clarification of the rate-limiting step in α-ketoglutarate reductive amination with NADPH and NH_4^+ (DiFranco and Iwatsubo, 1971). In the latter case the absorbance at 340 nm due to the nicotinamide ring is lost with chemical transformation so that the subsequent events are not observed by the absorption measurements used. It is thus difficult to interpret the linear decrease in absorbance seen with $NADP^+$ oxidation as compared with the burst kinetics observed with $NADP^+$ reduction, but the possibility of a rate-limiting step other than coenzyme dissociation, such as chemical transformation or glutamate dissociation, is not excluded by the transient kinetic study reported (DiFranco and Iwatsubo, 1971). Rate limitation of glutamate dissociation would seem somewhat less likely than the other possibilities due to the rate difference observed with NADH and NADPH (Table I).

The simultaneous fall after a maximum in both glutamate $\rightleftharpoons$ α-ketoglutarate and $NAD^+ \rightleftharpoons$ NADH rates with increasing glutamate:NH_4^+ concentration suggests that there may be a decrease in the number of chemically transformed molecules with time under these conditions (Silverstein, 1963; Boyer and Silverstein, 1963). If binding of glutamate and NH_4^+ occurs to the same site and is mutually exclusive, one would expect a rise to a sustained maximum with increasing glutamate:NH_4^+ concentration as forward and back reactions are facilitated by binding of glutamate and NH_4^+, respectively. This result should also obtain if NH_4^+ and glutamate may bind simultaneously at the catalytic center and there is no interference with chemical transformation. However, if such simultaneous binding would result in decreased chemical transformation at the chemical transformation step, perhaps by steric hindrance of required reactant alignment at the catalytic center or by interference with coenzyme binding to form a catalytically capable ternary complex, the result actually obtained would be expected. This analysis thus favors the possibility of simultaneous binding of glutamate and NH_4^+ to the catalytic center of bovine liver glutamate dehydrogenase.

This possibility is compatible with the apparently flexible or loose nature of the catalytic center which is suggested by the variety of substrates which can bind and be transformed there (Struck and Sizer, 1960; Tomkins *et al.*, 1965). It should be noted that NH_4^+ competitively inhibits the initial net rate of oxidative deamination of glutamate, suggesting that binding of the two ligands occurs at the same site (Fisher and McGregor, 1960). Other possible interpretations of those results are that binding of NH_4^+ at a site distinct from glutamate causes a decrease in the affinity of the enzyme for glutamate, or that there is a flexible site at the catalytic center capable of binding both ligands separately, which may be distorted into binding both simultaneously, a condition which would be favored by highly saturating levels of both glutamate and NH_4^+. Another theoretical possibility which cannot be totally excluded from the present data, but which would seem less likely, is low affinity binding of NH_4^+ to a site not at the catalytic center which would result either in decrease in the rate of the chemical transformation step, or in simultaneous and equal decrease in the dissociation steps which are rate limiting for $NAD^+ \rightleftharpoons$ NADH and glutamate $\rightleftharpoons$ α-ketoglutarate equilibrium reaction rates.

The presence of unreactive abortive complexes can be discovered with equilibrium kinetics by testing the effect on equilibrium rates of the concentration of a pair of reactants which cannot react with each other. The occupation of catalytic centers by small molecules which cannot react with each other effectively removes those enzyme molecules from catalysis and has the same effect as a decrease in enzyme concentration (Silverstein, 1963; Boyer and Silverstein, 1963). The present results thus suggest that glutamate and NADH can readily bind simultaneously to the enzyme and that α-ketoglutarate and NAD^+ can also do so to a lesser extent (Figures 5 and 6). Data suggesting the abortive complex enzyme–NADH–glutamate have also been obtained by initial net reaction kinetics (Engel and Dalziel, 1970a,b) and by absorbance (Egan and Dalziel, 1971). In the presence of the substrate analog glutarate, both NAD^+ and $NADP^+$ are bound more firmly to enzyme as determined by equilibrium dialysis (Dalziel and Egan, 1972), suggesting the analogous formation of the complex, enzyme–NAD^+–α-ketoglutarate, in agreement with the present experiments. While formation of the complex enzyme–NADP–α-ketoglutarate was not studied in the present experiments, cooperative binding of $NADP^+$ and α-ketoglutarate in a highly stable complex as determined by ultraviolet difference spectroscopy has been recently reported (Cross *et al.*, 1972).

There was relatively little disparity between coenzyme and substrate interchanges, especially with NAD^+, in contrast to more marked differences noted with other dehydrogenases (Silverstein and Boyer, 1964a,b; Silverstein and Sulebele,

1969b,c). This finding suggests that chemical transformation may be of the same order of magnitude as the slower reactant dissociation rates (Silverstein, 1963; Boyer and Silverstein, 1963). Relatively slow chemical transformation would seem reasonable for this dehydrogenase in view of the complexity of the 3–4 reactant enzyme reaction which includes (de) amination. It is not possible, however, to rule out that chemical transformation is much more rapid than rate-limiting dissociation rates which are fortuitously similar for the transforming reactant pairs.

Based on net initial rate kinetics with malate dehydrogenase (Harada and Wolfe, 1968a,b) and stop-flow kinetics with alcohol dehydrogenase (Bernhard *et al.*, 1970; McFarland and Bernhard, 1972), the interesting suggestion has been made that only a fraction of the catalytic centers of enzymes with subunits may be active in catalysis at any given time. Thus, the reciprocating mechanism (Harada and Wolfe, 1968a,b) includes chemical transformation at one of two catalytic sites only when the second has no bound reactants, and product dissociation from one catalytic center must occur before chemical transformation can occur at the other catalytic center. Analogous to previous experiments with malate, lactate, and alcohol dehydrogenases (Silverstein and Sulebele, 1969b,c; Silverstein and Boyer, 1964a,b) and yeast hexokinase (Fromm *et al.*, 1964) the present experiments do not lend support to a mechanism for bovine liver glutamate dehydrogenase in which product at one catalytic center must dissociate before catalysis can occur at another (McFarland and Bernhard, 1972) since substrate interchange was not surpressed by increasingly elevated glutamate: α-ketoglutarate which would tend to saturate all subunit substrate sites. However, a model which does not require substrate dissociation at one catalytic center for chemical transformation at another (Bernhard *et al.*, 1970) would not be incompatible with the present experiments. The finding of stronger binding of coenzyme at low coenzyme concentrations than at higher concentrations by equilibrium dialysis in the presence of glutamate has suggested that there either are negative homotropic interactions between catalytic centers in the binding of coenzyme in ternary complexes with substrate or that the six subunits are not identical (Dalziel and Egan, 1972).

References

Bernhard, S. A., Dunn, M. F., Luisi, P.-L., and Schack, P. (1970), *Biochemistry 9*, 185.

Boyer, P. D. (1959), *Arch. Biochem. Biophys. 82*, 387.

Boyer, P. D., and Silverstein, E. (1963), *Acta Chem. Scand. 17*, S195.

Cassman, M., and Schachman, H. (1971), *Biochemistry 10*, 1015.

Colen, A. H., Prough, R. A., and Fisher, H. F. (1972), *J. Biol. Chem. 247*, 7905.

Corman, L., Prescott, L. M., and Kaplan, N. O. (1967), *J. Biol. Chem. 242*, 1383.

Cross, D. G. (1972), *J. Biol. Chem. 247*, 784.

Cross, D. G., McGregor, L. L., and Fisher, H. F. (1972), *Biochim. Biophys. Acta 289*, 28.

Dalziel, K., and Egan, R. R. (1972), *Biochem. J. 126*, 975.

DiFranco, A., and Iwatsubo, M. (1971), *Biochimie 53*, 153.

Egan, R. R., and Dalziel, K. (1971), *Biochim. Biophys. Acta 250*, 47.

Eisenberg, H. (1970), *in* Pyridine Nucleotide Dependent Dehydrogenases, Sund, H., Ed., West Berlin, Springer Verlag, p 293.

Engel, P. C., and Dalziel, K. (1969), *Biochem. J. 115*, 621.

Engel, P. C., and Dalziel, K. (1970a), *Biochem. J. 118*, 409.

Engel, P. C., and Dalziel, K. (1970b), *in* Pyridine Nucleotide Dependent Dehydrogenases, Sund, H., Ed., West Berlin, Springer-Verlag, 1970, p 245.

Fahien, L. A., and Strmecki, M. (1969), *Arch. Biochem. Biophys. 130*, 468.

Fisher, H., and McGregor, L. (1960), *Biochem. Biophys. Res. Commun. 3*, 629.

Fisher, H. F., Bard, J. R., and Prough, R. A. (1970), *Biochem. Biophys. Res. Commun. 41*, 601.

Frieden, C. (1959a), *J. Biol. Chem. 234*, 809.

Frieden, C. (1959b), *J. Biol. Chem. 234*, 2891.

Frieden, C. (1963), *Enzymes, 2nd Ed., 7*, 3.

Frieden, C. (1964), *Brookhaven Symp. Biol. 17*, 98.

Frieden, C. (1968), *in* The Role of Nucleotides for the Function and Conformation of Enzymes, Kalckar, H. M., Klenow, H., Munch-Peterson, A., Otteseon, M., and Thayson, J. H., Ed., Copenhagen, Munksgaard, p 194.

Fromm, H. J., Silverstein, E., and Boyer, P. D. (1964), *J. Biol. Chem. 239*, 3645.

Harada, K., and Wolfe, R. G. (1968a), *J. Biol. Chem. 243*, 4123.

Harada, K., and Wolfe, R. G. (1968b), *J. Biol. Chem. 243*, 4131.

Horecker, B. L., and Kornberg, A. (1948), *J. Biol. Chem. 175*, 385.

Iwatsubo, M., and Pantaloni, D. (1967), *Bull. Soc. Chim. Biol. 49*, 1563.

Jallon, J.-M., and Iwatsubo, M. (1971), *Biochem. Biophys. Res. Commun. 45*, 964.

Josephs, R., and Borisy, G. (1972), *J. Mol. Biol. 65*, 127.

Markau, K., Schneider, J., and Sund, H. (1971), *Eur. J. Biochem. 24*, 393.

McFarland, J. T., and Bernhard, S. A. (1972), *Biochemistry 11*, 1486.

Pantaloni, D., and Dessen, P. (1969), *Eur. J. Biochem. 11*, 510.

Prough, R. A., Colen, A. H., and Fisher, H. F. (1972a), *Biochim. Biophys. Acta 284*, 16.

Prough, R. A., Culver, J. M., and Fisher, H. F. (1972b), *Arch. Biochem. Biophys. 149*, 414.

Silverstein, E. (1963), Ph.D. Thesis, University of Minnesota.

Silverstein, E. (1965), *Anal. Biochem. 12*, 199.

Silverstein, E. (1968), 156th National Meeting of the American Chemical Society, Atlantic City, N. J., Sept, Abstract BIOL-109.

Silverstein, E. (1970a), *Biochim. Biophys. Acta 198*, 148.

Silverstein, E. (1970b), 160th National Meeting of the American Chemical Society, Chicago, Ill., Sept, Abstract BIOL-49.

Silverstein, E. (1970c), *Proc. Int. Congr. Biochem., 8th, Lucerne, 1970, Symp. VII*, Session A, No. 7.

Silverstein, E., and Boyer, P. D. (1964a), *J. Biol. Chem. 239*, 3901.

Silverstein, E., and Boyer, P. D. (1964b), *J. Biol. Chem. 239*, 3908.

Silverstein, E., and Boyer, P. D. (1966), *Biochem. Prep. 11*, 89.

Silverstein, E., and Sulebele, G. (1966), 152nd National Meeting of the American Chemical Society, New York, N. Y., Sept, Abstract BIOL-124.

Silverstein, E., and Sulebele, G. (1967), *Proc. Int. Congr. Biochem., 7th, 1967*, F 303.

Silverstein, E., and Sulebele, G. (1969a), 158th National Meeting of the American Chemical Society, New York, N. Y., Sept, Abstract BIOL-232.

Silverstein, E., and Sulebele, G. (1969b), *Biochemistry 8*, 2543.
Silverstein, E., and Sulebele, G. (1969c), *Biochim. Biophys. Acta 185*, 297.
Silverstein, E., and Sulebele, G. (1969d), 158th National Meeting of the American Chemical Society, New York, N. Y., Sept, Abstract BIOL-261.
Silverstein, E., and Sulebele, G. (1970a), *Biophys. J. 10*, 123a.
Silverstein, E., and Sulebele, G. (1970b), *Biochemistry 9*, 274, 283.
Smith, E. L., Landon, M., Piszkiewica, D., Brattin, W. J., Langley, T. J. and Melamed, M. D. (1970), *Proc. Nat. Acad. Sci. U. S. 67*, 724.
Struck, J., and Sizer, I. W. (1960), *Arch. Biochem. Biophys. 86*, 260.
Tomkins, G. M., Yielding, K. L., Curran, J. F., Summers, M. R., and Bitensky, M. W. (1965), *J. Biol. Chem. 240*, 3793.

Affinity Labeling of the Active Sites of Δ^5-Ketosteroid Isomerase Using Photoexcited Natural Ligands†

Richard J. Martyr‡ and William F. Benisek*

ABSTRACT: In attempts to use natural ligands which bind to macromolecules as their own affinity reagents, we have explored the use of photoexcited ketones. The activity of Δ^5-3-ketosteroid isomerase of *Pseudomonas testosteroni* is only slightly reduced when the enzyme is irradiated with ultraviolet light of wavelengths above 300 nm unless steroid ketones which are competitive inhibitors of the enzyme are present. The order of effectiveness of steroid ketones in promoting photoinactivation is 3-oxo-4-estren-17β-yl acetate > 17β-hydroxy-4-estren-3-one > 17β-hydroxy-4-androsten-3-one $\gg$ 1-cyclohexen-2-one, which parallels the order of affinity of these substances for the enzyme's active site. The competitive inhibitors 3β-hydroxy-5-pregnen-20-one and 3-methoxy-1,3,5(10)-estratrien-17β-ol do not support photoinactivation at a significant rate. The inactivation promoted by 3-oxo-4-estren-17β-yl acetate is slowed in the presence of the competitive inhibitor 3β-hydroxy-5-androstene-17β-carboxylic acid. Sephadex chromatography of isomerase photoinactivated in the presence of 4-[^{14}C]-3-oxo-4-estren-17β-yl acetate showed that radioactivity became associated with the protein during the photoinactivation, whereas enzyme which was incubated with the steroid in the dark did not become associated with appreciable radioactive material. These results suggest that photoinactivation proceeds by excitation of the keto group of an active-site bound 3-keto steroid followed by chemical reactions between enzyme functional groups and the electronically excited ketone. One or more of these reactions may involve covalent attachment of the steroid to the enzyme.

Affinity labeling of the binding sites for small molecules (ligand binding sites) on protein molecules has in numerous instances helped to identify functional group components of such sites or to identify which protein in a complex mixture of proteins contained the site of interest (Shaw, 1970). The accuracy of detailed structural interpretations based on affinity labeling is contingent upon two conditions. The first is that the site which is labeled by the affinity reagent is the same as that which binds the natural ligand. The second is that the affinity reagent noncovalently binds to the site congruently with the mode of binding of the natural ligand.

Frequently, grafting of a chemically reactive functional group onto the molecular structure of the natural ligand in order to produce a potential affinity reagent results in a sufficiently substantial structural alteration such that either one or both of the conditions mentioned may not be met.

One could avoid the possibility of not satisfying these conditions if one employed the natural ligand as its own affinity reagent. Usually this is not feasible since the natural ligand does not contain a suitably chemically reactive group. However, many natural ligands including substrates, allosteric effectors, and hormones contain functional groups which, when promoted to electronically excited states by absorption of light of appropriate wavelength, are converted from chemically inert to chemically reactive species. Ligand molecules which contain such functional groups could then function as their own affinity reagents when illuminated with radiation of the correct wavelengths. Affinity labeling based on electronic excitation of the natural ligand would, then, be a three-step process (Scheme I): (1) noncovalent binding of the natural

SCHEME I

$$\text{protein} + \text{ligand} \rightleftharpoons \text{protein}\cdot\text{ligand}$$

$$\text{protein}\cdot\text{ligand} \overset{h\nu}{\rightleftharpoons} \text{protein}\cdot\text{ligand}^*$$

$$\text{protein}\cdot\text{ligand}^* \longrightarrow \text{chemically modified protein}$$

ligand to its appropriate site on a macromolecule; (2) absorption of light by the bound ligand resulting in its electronic excitation; (3) reaction of the excited ligand with a functional group(s) in its binding site.

Ligand molecules which contain ketone groups possess the properties which could allow them to be used as affinity reagents. When irradiated with light in the wavelength range 280–320 nm, ketones are excited to a diradical-like state which

† From the Department of Biological Chemistry, School of Medicine, University of California, Davis, California 95616. *Received February 12, 1973.* Supported by a grant from the National Institutes of Health (AM-14729).

‡ Supported by National Institutes of Health Predoctoral Traineeship GM-119-14.