Johnstone, R. M. (1976) *Amino Acid Transp. Uric Acid Transp., Symp. Innsbruck, 1975*, 12–19.
Kilberg, M. S., & Christensen, H. N. (1979) *Biochemistry 18*, 1525–1530.
Low, H., & Crane, F. L. (1978) *Biochim. Biophys. Acta 515*, 141–161.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Mitchell, M. E., Aronson, P. S., & Sacktor, B. (1974) *J. Biol. Chem. 249*, 6971–6975.
Murer, H., Hopfer, V., & Kinne, R. (1976) *Biochem. J. 154*, 597–604.
Ramos, S., & Kaback, H. R. (1977) *Biochemistry 16*, 848–854.
Sacktor, B. (1977) in *Current Topics of Bioenergetics* (Rao Sanadi, D., Ed.) pp 39–81, Academic Press, New York.
Schafer, J. A., Richey, B. E., & Williams, A. E. (1977) *J. Gen. Physiol. 69*, 681–704.
Thuneberg, L., & Rostgaard, J. (1968) *Exp. Cell Res. 51*, 123–140.

Kinetic Mechanism of Horse Liver Alcohol Dehydrogenase SS†

Casimir N. Ryzewski‡ and Regina Pietruszko*

ABSTRACT: The kinetic mechanism of SS isozyme of horse liver alcohol dehydrogenase is shown by initial velocity and product inhibition studies to be asymmetrical, being random for ethanol oxidation and compulsory ordered for acetaldehyde reduction. Enzyme isomerization seems to account for the asymmetry in the mechanism. In its interaction with NADH, the SS isozyme resembles classical alcohol dehydrogenase; consequently, the maximal velocity in the direction from ethanol to acetaldehyde appears to be determined by the rate of NADH dissociation. In the direction from acetaldehyde to ethanol, the enzyme isomerization step appears to limit the maximal velocity.

The kinetic mechanism of classical horse liver alcohol dehydrogenase [a preparation containing 60–80% of EE isozyme[1] and henceforth referred to as (EE) isozyme] was determined by several investigators (Theorell & Chance, 1951; Theorell & McKinley-McKee, 1961; Dalziel, 1957; Dalziel & Dickinson, 1966a,b) and that of homgeneous EE isozyme was determined by Hanes et al. (1972). The kinetic mechanism of (EE) isozyme is best described as a special case of a symmetrical ordered bi-bi mechanism in which the dissociation of the product–coenzyme, rather than the interconversion of the productive ternary complexes, is rate limiting (Cleland, 1970; Dalziel, 1975). The formation of the productive ternary complexes has been verified experimentally by Wratten & Cleland (1963, 1965), Theorell & Yonetani (1962), and Theorell & Tatemoto (1970), the latter employing homogeneous EE isozyme. The mechanism deviates from the compulsory ordered mechanism when initial velocity studies are performed with secondary alcohols (Dalziel & Dickinson, 1966b). Evidence for alcohol oxidation via a partially random mechanism was found in isotope exchange studies (Silverstein & Boyer, 1964) and in initial velocity studies using a wide range of substrate concentrations (Hanes et al., 1972). Hence, the kinetic mechanism of EE isozyme can be more precisely described as partially random for alcohol oxidation and compulsory ordered for aldehyde reduction with the rate of product–coenzyme dissociation as the rate-limiting step.[2]

The EE and SS isozymes are well suited for structure–function studies in view of the small amino acid sequence difference between the E and S subunits (Jörnvall, 1970a,b). Both subunits when present as a part of a dimeric enzyme molecule possess the wide substrate specificity which is characteristic of alcohol dehydrogenase, but only the S subunit can catalyze reversible redox reactions between 3-ketosteroids and 3β-hydroxysteroids (Pietruszko et al., 1966; Theorell et al., 1966). The steroid activity appears to be the result of a single amino acid difference between the E and S subunits (Eklund et al., 1976). The kinetic mechanism of the acetaldehyde–ethanol interconversion catalyzed by SS isozyme has not been determined.[3] Elucidation of this functional aspect of SS isozyme is necessary for comparison with EE isozyme and for determining structure–function relationships between these isozymes.

Experimental Procedures

NADH (grade III) was purchased from Sigma Chemical Co., St. Louis, MO. NAD (grade II) obtained from Boeh-

† From the Center of Alcohol Studies, Rutgers University, New Brunswick, New Jersey 08903. *Received October 11, 1979.* Research funded by USPHS–NIAAA Grant AA00186, the Charles Johanna Busch Memorial Fund, and Research Scientist Development Award K02-AA00046 to R.P.

‡ Present address: Department of Molecular and Cellular Developmental Biology, University of Colorado, Boulder, CO 80309.

[1] Abbreviations used: NAD, nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; (EE) isozymes, classical preparations of horse liver alcohol dehydrogenase; EE and SS isozymes, single, isozyme preparations; ϕ_O, ϕ_A, ϕ_B, and ϕ_{AB}, kinetic coefficients referring to the reaction in the direction from ethanol to acetaldehyde; ϕ'_O, ϕ_Q, ϕ_P, and ϕ_{PQ}, kinetic coefficients referring to aldehyde reduction. Cleland nomenclature is employed throughout: K_a, K_m(NAD); K_{ia}, dissociation constant for NAD from the enzyme·NAD binary complex; K_b, K_m(ethanol); K_{ib}, dissociation constant for ethanol from the enzyme·ethanol binary complex; V_1, turnover number × (active site)$^{-1}$ × s^{-1} at saturating NAD and ethanol. K_q, K_m(NADH); K_{iq}, dissociation constant for NADH from the enzyme·NADH binary complex; K_p, K_m(acetaldehyde); V_2, turnover number × (active site)$^{-1}$ × s^{-1} at saturating NADH and acetaldehyde. V_1/(Et) and V_2/(Et) refer to saturating ethanol and acetaldehyde at 500 μM NAD and 170 μM NADH.

[2] This work is a part of a Ph.D. thesis of C. N. Ryzewski.

[3] The catalytic mechanism of an isozyme of alcohol dehydrogenase described as SS isozyme has been published by Dworschack & Plapp (1977). The properties and mechanism of their enzyme are so different from ours that to save space we have decided not to compare our data with theirs. We also possess evidence (C. N. Ryzewski and R. Pietruszko, unpublished experiments) proving that their and our data refer to different isozymes.

0006-2960/80/0419-4843$01.00/0

Table I: Kinetic Coefficients and Kinetic Constants from Initial Velocity Studies

kinetic coefficients at pH 7.0 calcd by the method of Dalziel[a]			kinetic constants calcd from ϕ coefficients				
	ϕ coeff					kinetic constant	
ϕ coeff	SS isozyme	(EE) isozyme[b]	calcd from ϕ coeff ratio	substrate	kinetic constant	SS isozyme	(EE) isozyme
			Ethanol Oxidation				
ϕ_O (s)	0.82	0.37	$1/\phi_O$		V_1 (s^{-1})	1.2	2.7
ϕ_A (s μM)	29.1	1.1	ϕ_A/ϕ_O	NAD	K_a (μM)	35.5	3.0
ϕ_B (s μM)	94000	66	ϕ_B/ϕ_O	ethanol	K_b (μM)	114800	178
ϕ_{AB} (s μM^2)	2520000	7200	ϕ_{AB}/ϕ_B	NAD	K_{ia} (μM)	26.8	109.1
			ϕ_{AB}/ϕ_A	ethanol	K_{ib} (μM)	86600	–
			Acetaldehyde Oxidation				
ϕ'_O (s)	0.28	0.008	$1/\phi'_O$		V_2 (s^{-1})	35.7	125
ϕ_P (s μM)	220	3.3	ϕ_P/ϕ'_O	acetaldehyde	K_p (μM)	7800	412
ϕ_Q (s μM)	0.12	0.1	ϕ_Q/ϕ'_O	NADH	K_q (μM)	4.4	12.5
ϕ_{PQ} (s μM^2)	126	1.4	ϕ_{PQ}/ϕ_P	NADH	K_{iq} (μM)	0.57	0.42

[a] Dalziel (1957). [b] Dalziel (1936b).

ringer Mannheim Corp., New York, NY, was purified by the method of Dalziel (1963a) and was stored frozen. Ethanol was a product of the Commercial Solvent Corp., Terre Haute, IN. Acetaldehyde purchased from Matheson Coleman and Bell, Norwood, OH, was redistilled prior to use each day.

Homogeneous SS isozyme was prepared from horse livers as previously described (Ryzewski & Pietruszko, 1977). The enzyme, stored in 20% glycerol in 0.1 M sodium phosphate, pH 7.0, at 4 °C, was dialyzed with at least three changes of 2000 times its volume of 0.1 M sodium phosphate, pH 7.0, prior to use. The enzyme was found to be stable for the duration of each experimental period as determined by assaying its activity with cyclohexanone (12.3 mM) and NADH (170 μM) in 0.1 M sodium phosphate, pH 7.0.

Enzyme normality was determined by fluorometric titration with NADH in the presence of 0.1 M isobutyramide (Winer & Theorell, 1960) using an Aminco-Bowman spectrofluorometer.

All kinetic determinations were performed in 0.1 M sodium phosphate, pH 7.0, at 25 °C in final assay volumes of 3 mL. Reactions were followed by measuring the appearance or disappearance of NADH at 340 nm in cuvettes with a 1-cm light path by employing a Varian Instruments Model 635 spectrophotometer or a Cary Model 118 spectrophotometer at 1:50 scale expansion for measuring small changes of optical density. The reaction progress curves were linear.

Lineweaver & Burk (1934) plots of initial velocities were obtained by the weighted method of Wilkinson (1961). Each data point for an initial velocity determination represents the average of at least two determinations which did not differ from the average by more than 5%. Data points which differed by more than 5% were repeated or excluded. Linear replots of the kinetic data were performed by the method of least squares. Student's t test[4] was used to determine p values to show if differences between intercepts or between slopes were significant when small differences were encountered in product inhibition patterns.

Results and Discussion

Alcohol dehydrogenase from horse liver, since its crystallization by Bonnichsen & Wassén (1948) and the pioneering studies of Theorell & Chance (1951), has served as a model enzyme for kinetics. Consequently, an appreciable amount of information about the catalytic mechanism of SS isozyme can be obtained by comparison with the data available on (EE) isozyme.

Initial Velocity Studies of SS Isozyme at pH 7. The mechanism of the acetaldehyde–ethanol interconversion by SS isozyme was first investigated by steady-state initial velocity methods. All primary Lineweaver–Burk plots over the substrate and coenzyme concentration ranges tested were linear and intersected in the third quadrant. The ϕ coefficients (Dalziel, 1957) were calculated from replots of the slopes and intercepts vs. the inverse of the concentration of the substrate or coenzyme reaction partner considered to be constant. All replots were also linear.

The calculated ϕ coefficients for SS isozyme are presented in Table I and compared to those determined by Dalziel (1963b) for (EE) isozyme. Two values were calculated for each coefficient depending on which substrate or coenzyme was considered as constant, and the average of the two coefficients is shown in Table I. With the exception of ϕ_Q (almost identical for both isozymes) and ϕ_O and ϕ'_O (which are not greatly different), the ϕ values for SS and (EE) isozymes differ by more than 1 order of magnitude.

The ϕ coefficients for SS isozyme were used to calculate kinetic constants which are also compared with those of Dalziel (1963b) for (EE) isozyme in Table I. The most striking difference between SS and (EE) isozymes is in constants relating to substrate binding (compare K_b and K_p values); there are also major differences in K_p K_a and K_{ia} values.

The S and E subunits differ in only 6 amino acid residues in a total of 374 (Jörnvall, 1970a, b). X-ray crystallographic work (Brändén et al., 1975) has demonstrated that two of these residues (110 and 115) are located within the substrate binding area of the active site. It is therefore not surprising to find that the major differences between SS and (EE) isozymes are in constants relating to substrate binding. Differences in K_a and K_{ia} values (relating to NAD binding) are, however, unexpected as sequence studies do not show any differences between the coenzyme-binding sites of EE and SS isozymes.

The intersecting pattern of Lineweaver–Burk plots giving values of ϕ_{AB} and ϕ_{PQ} which are greater than zero shows that the kinetic mechanism of SS isozyme is sequential like that of (EE) isozyme and of other pyridine nucleotide dependent dehydrogenases. The ratio of $(\phi_{PQ}/\phi_{AB}) \times 10^{-7}$ gives a value of 5×10^{-12} for the equilibrium constant of the overall reaction. This compares well with that determined directly [8×10^{-12}; Bäcklin (1958)] and indicates that the data obtained here from

[4] p = statistical probability of finding differences due to chance alone; the smaller the p value the greater is the chance of a real difference between the experimental variants.

Table II: Comparison of Dalziel[a] Coefficient Ratios for Calculating Coenzyme Dissociation Constants and of Dalziel[b] Maximal Rate Relations for SS and (EE) Isozymes

ϕ coeff ratios	alcohol dehydrogenase isozymes SS[c]	(EE)[d]	definitions of mechanism
(A) Dissociation Constants for Coenzyme			
(1) Theorell-Chance Mechanism[a]			
(a) ϕ_{PQ}/ϕ_P (for NADH)	0.57	0.42	
(b) ϕ_Q/ϕ_O (for NADH)	0.15	0.27	(a) = (b)
(c) ϕ_{AB}/ϕ_B (for NAD)	26.8	109.1	
(d) ϕ_A/ϕ'_O (for NAD)	1039	137.5	(c) = (d)
(2) Random Mechanism-Noninteracting[a]			
(e) ϕ_{PQ}/ϕ_P (for NADH)	0.57	0.42	
(f) ϕ_Q/ϕ'_O (for NADH)	4.4	12.5	(e) = (f)
(g) ϕ_{AB}/ϕ_B (for NAD)	26.8	109.1	
(h) ϕ_A/ϕ_O (for NAD)	35.5	35.5	(g) = (h)
(B) Maximal Rate Relations for a Compulsory Ordered Mechanism			
(i) $\phi_{PQ}/(\phi_P\phi_Q)$ (s^{-1})	4.7	4.2	
(j) $1/\phi_O$ (s^{-1})	1.2	2.7	(i) = (j)
(k) $\phi_P\phi_Q/\phi_{PQ}\phi_O$	0.26	0.6	(k) = 1
(l) $\phi_{AB}/(\phi_A\phi_B)$ (s^{-1})	0.92	99	
(m) $1/\phi'_O$ (s^{-1})	35.7	125	(l) = (m)
(n) $\phi_A\phi_B/(\phi_{AB}\phi'_O)$	38.8	1.3	(n) = 1

[a] Dalziel (1957). [b] Dalziel (1975). [c] Our data. [d] Dalziel (1963b). The ratios (k) and (n) cannot exceed unity for a simple ordered mechanism; values close to unity indicate that product coenzyme dissociation determines the rate; values much smaller than unit indicate that some other step is rate limiting. Large values are inconsistent with a simple ordered mechanism.

initial velocity studies are reliable.

Kinetic coefficients ϕ_Q for SS and (EE) isozymes are almost identical. These coefficients relate to interaction with NADH, and their nearly identical values suggest that SS and (EE) isozymes interact with NADH in a similar, if not identical, manner. The reciprocals of ϕ_Q give rate constants for NADH binding of 10 s^{-1} μM^{-1} and 8.2 s^{-1} μM^{-1} for (EE) and SS isozymes, respectively. However, comparison of ϕ_A coefficients relating to the interaction of these isozymes with NAD does not lead to similar conclusions, since these coefficients differ by more than 1 order of magnitude.

The ratios of ϕ coefficients used for calculating coenzyme dissociation constants shown in Table I hold for compulsory ordered and rapid equilibrium random mechanisms. Additional ratios of ϕ coefficients are used to distinguish between variants within mechanisms (Dalziel, 1957). For a compulsory ordered mechanism in which ternary complexes are not kinetically significant (e.g., Theorell–Chance mechanism) or for a rapid equilibrium random mechanism in which binding of one ligand does not alter the affinity for another ligand, there are additional ratios of ϕ coefficients for calculating coenzyme dissociation constants. The two ratios of ϕ coefficients for each coenzyme defining the Theorell–Chance mechanism and the random order noninteracting mechanism are compared for (EE) and SS isozymes in Table II. The data for (EE) isozyme are consistent with the Theorell–Chance mechanism in that (a) = (b) and (c) = (d). The data for SS isozyme resemble those for (EE) isozyme in the values calculated for NADH where (a) is similar to (b) in magnitude. The values of the ratios of ϕ coefficients for NAD are quite different for (EE) and SS isozymes. While (c) equals (d) for (EE) isozyme, for SS isozyme (c) differs from (d) by more than 1 order of magnitude, precluding Theorell–Chance mechanism. The ratios of ϕ coefficients for NAD for SS isozyme are more consistent with the random mechanism (noninteracting) where ratios (g) and (h) approach equality.

Table II also shows the Dalziel (1975) maximal rate relations for both (EE) and SS isozymes. The values of relationships (i), (j), and (k) for NADH–acetaldehyde are similar for both isozymes. Differences between (EE) and SS isozymes are apparent by comparison of maximal rate relations for NAD–ethanol. The value of relationship (l) is 2 orders of magnitude smaller for SS than for (EE) isozyme, and relationship (n) is almost 30 times larger for SS than for (EE) isozyme. According to Dalziel (1975) relationships (k) and (n) cannot exceed unity for a simple ordered symmetrical mechanism. The value of relationship (k) is similar for (EE) and SS isozymes and is less than unity (but not considerably less than unity) in both cases, a value consistent with the ordered mechanism for the reduction of acetaldehyde by both isozymes. The value of relationship (n) for (EE) isozyme exceeds unity only slightly (Table II) and is consistent with an ordered mechanism, but for SS isozyme it is 38.8 times larger than unity. This large value of relationship (n) is inconsistent with the ordered mechanism for ethanol oxidation by SS isozyme.

It can be seen in Table II that for (EE) isozyme relationships (l) and (m) are equal within the experimental error of the procedure employed, while there is 40-fold discrepancy between relationships (l) and (m) for SS isozyme. The large numerical discrepancy between relationships (l) and (m) indicates that the rate-limiting step for the reduction of acetaldehyde by SS isozyme must be other than the dissociation of the enzyme·NAD binary complex.

Comparison of Kinetic Constants for Ethanol and Oxidation and Off Velocity Constants for NADH. In Table III, Michaelis constants for ethanol [K_m(apparent) at 500 μM NAD] and catalytic rate constants V_1/(Et) [turnover number × (active site)$^{-1}$ × s^{-1} for ethanol oxidation at 500 μM NAD]

Table III: Michaelis and Catalytic Rate Constants of SS Isozyme at Different pH Values, Comparison with Dissociation of the S·NADH Binary Complex, and Michaelis and Catalytic Rate Constants of (EE) Isozyme[a]

SS isozyme[b]			SS isozyme[c]			(EE) isozyme		
pH	K_m (mM)	V_1/(Et)	pH	k_{off}/s	K_D(NADH) (μM)	pH	K_m[d] (mM)	V_1[e]
6.1	118	1.0	6.0	2.1	0.08	6.0	2.5	1.6
7.0	42	1.2	7.0	1.4	0.09	7.1	0.59	2.7
7.6	22	1.6	8.0	1.3	0.09	8.0	0.25	3.2
8.8	16	2.0	9.0	2.1	0.16	9.0	0.60	3.8
10.0	7	3.6	10.0	3.9	0.52	10.0	2.0	5.2

[a] Our assay system contained the following: 500 μM NAD and 0.1 M sodium phosphate at pH 6.1, 7.0, and 7.6; at pH 8.8 0.1 M sodium pyrophosphate was used, and 0.065 M glycine–NaOH buffer was employed at pH 10.0. Each pH was determined after completion of rate determination. The reaction was carried out in cuvettes of 1-cm light path at 340 nm and 25 °C. k_{off}/s = the rate of dissociation of the S·NADH binary complex, determined directly by fluorometry; V_1 = turnover number × (active site)$^{-1}$ × s^{-1} at a saturating concentration of ethanol and NAD; V_1/(Et) = turnover number × (active site)$^{-1}$ × s^{-1} at saturating ethanol and 500 μM NAD. [b] Our data. [c] Theorell et al. (1970). [d] Sund & Theorell (1963). [e] Dalziel (1975).

Table IV: Summary of the Product Inhibition Studies for SS Isozyme

constant substrate (mM)	product inhibitor	variable substrate	type of inhibn	K_i (μM)
NAD (0.5)	NADH	ethanol	competitive	6.5[a]
ethanol (292)	NADH	NAD	competitive	8.6[a]
NAD (0.5)	acetaldehyde	ethanol	competitive	280
ethanol (292)	acetaldehyde	NAD	competitive	240
acetaldehyde (60)	NAD	NADH	competitive	20
NADH (0.17)	NAD	acetaldehyde	noncompetitive	1360 slope 307 intercept
acetaldehyde (60)	ethanol	NADH	noncompetitive	parabolic slope 607 000 intercept
NADH (0.17)	ethanol	acetaldehyde	noncompetitive	parabolic slope parabolic intercept

[a] When this value was redetermined at 10.8 mM ethanol (a concentration about one-tenth of the K_m value), it was 0.55 μM.

are compared as a function of pH with values obtained in similar conditions by other investigators for (EE) isozyme. The *off* velocity constants for NADH dissociation from SS isozyme and dissociation constants (K_D) for the S·NADH binary complex determined directly by Theorell et al. (1970) are also listed in Table III.

The Michaelis constants for ethanol for SS isozyme at all pH values are 1–2 orders of magnitude larger than those for (EE) isozyme. The Michaelis constants for ethanol for SS isozyme decrease with an increase of pH; the K_m at pH 10 is relatively small, ~17% of that at pH 7.0. The Michaelis constants for (EE) isozyme show a minimum at pH 8.0, and the K_m value for ethanol at pH 10 is relatively large, ~340% of that at pH 7.0. The V_1/(Et) values for SS isozyme, although somewhat lower than V_1 for (EE) isozyme, vary with pH in a way analogous to (EE) isozyme.

Comparison of V_1/(Et) values for SS isozyme with off velocity constants for NADH from the S·NADH binary complex shows that they are almost identical (with possible exception of the value at pH 6.1). This suggests strongly that for ethanol oxidation by the SS isozyme the rate-limiting step is the dissociation of the enzyme·NADH binary complex.

The above results suggest that the catalytic mechanism of acetaldehyde reduction by SS isozyme is different from that of ethanol oxidation and that the rate-limiting step for ethanol oxidation is different from that for acetaldehyde reduction. In the direction from ethanol to acetaldehyde, the rate-limiting step is dissociation of NADH, as demonstrated by comparison of V_1/(Et) values with the off velocity constants for NADH from the S·NADH binary complex (Table III) and by Dalziel maximal rate relationships (Table II). For the reduction of acetaldehyde, the rate of coenzyme dissociation cannot be limiting the maximal velocity, as indicated by the large numerical discrepancy between relationships (l) and (m) in Table II.

Dalziel (1975) has pointed out that maximal rate relations can also yield information about isomerization of the enzyme. Isomerization is indicated when relationships (i) and (l) are small relative to relationships (j) and (m), respectively. For SS isozyme the relationship (l) is almost 40 times less than the maximal velocity of the reverse reaction (m) and 1 order of magnitude less than the rate constant for NADH binding, suggesting that isomerization of the SS isozyme might be the rate-limiting step for the reduction of acetaldehyde.

In our previous publication (Ryzewski & Pietruszko, 1977), we reported apparent K_m and V_2/(Et) values [turnover number × (active site)$^{-1}$ × s^{-1} at saturating aldehyde and 170 μM NADH] for SS isozyme with aldehydes of two to six carbon chain length. The V_2/(Et) values for the whole series (with the possible exception of hexanaldehyde) were similar with an average of 34.6/s for two, three, four, and five carbon chain length aldehydes, a value virtually identical with that found here for acetaldehyde (35.7/s). The three steps of the catalytic sequence which could have substrate-independent velocity are coenzyme binding and dissociation and enzyme isomerization. Since the *on* velocity constant for NADH is at least 8.2 s^{-1} μM^{-1} and NAD dissociation does not appear to be the rate-limiting step, it appears likely that the maximal velocity is limited by enzyme isomerization.

Product Inhibition Studies with SS Isozyme at pH 7.0. Product inhibition studies were performed, and a summary of the type of inhibition and the K_i values is presented in Table IV. When either ethanol or NAD was varied, all product inhibition patterns were competitive (p = 56–98% for intercepts). In contrast, only the product inhibition pattern when NADH was varied and NAD was product inhibitor was competitive (p^4 = 19–34% for intercepts). Noncompetitive product inhibition patterns were found when NADH was varied and ethanol was the inhibitor (p = 0.5–1.1% for intercepts) and when acetaldehyde was varied and ethanol was the inhibitor (p = 0.2–4.7% for intercepts). Lineweaver–Burk plots when acetaldehyde was varied and NAD was the inhibitor appeared to be parallel with a definite intercept effect; however, slopes for the plots at the two highest concentrations of NAD were found to be significantly different (p = 2%) from that of the control, and at the lowest concentration of NAD the difference in the slope from that of the control was just outside 95% confidence limits (p = 6.5%). The inhibition is therefore considered to be noncompetitive. Linear product inhibition replots were found for all cases except for the slope replots of ethanol inhibition when NADH was varied and for the slope and intercept replots of ethanol inhibition when acetaldehdye was varied. The replots were parabolic at the (292 mM) ethanol concentration used (Table IV); at 100 mM ethanol no inhibition was detected.

Product inhibition patterns for SS isozyme (Table IV) confirm the asymmetry observed in initial velocity studies. It is apparent that the mechanism by which SS isozyme oxidizes ethanol (four competitive patterns) differs from the mechanism by which it reduces acetaldehyde (three noncompetitive and one competitive pattern).

The patterns for the NADH–acetaldehyde side of the reaction are consistent with an ordered mechanism in which NADH binds to the enzyme first. NAD is a competitive product inhibitor when NADH is varied, giving a K_{ia} of 20 μM, comparable to that from initial velocity studies. Two values of an inhibition constant were calculated for NAD when acetaldehyde was varied and inhibition by NAD was found to be noncompetitive. The larger value was calculated from a slope replot where experimental error is large due to small differences between slopes; the smaller (more accurate) value was obtained from the intercept replot. Both values are

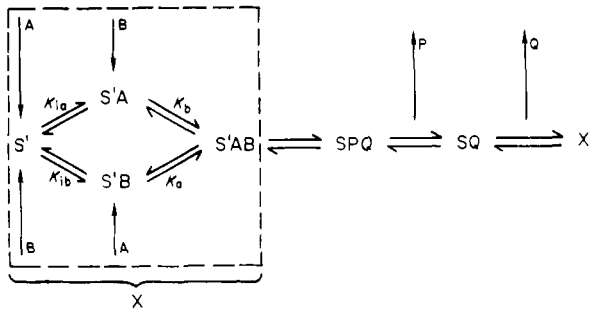

FIGURE 1: A scheme for the proposed kinetic mechanism of SS isozyme. A = NAD; B = ethanol; P = acetaldehyde; Q = NADH; S′ and S = isomeric forms of the SS isozyme.

consistent with an ordered mechanism and indicate formation of the dead-end ternary complex enzyme·NAD·acetaldehyde. Inhibition patterns with ethanol as product inhibitor are difficult to interpret since concentrations of ethanol are large and nonspecific solvent effects may be responsible for parabolic slope and intercept replots.

The patterns for the ethanol–NAD side of the reaction are all competitive, suggesting a random mechanism for ethanol oxidation by SS isozyme. The large vlaue of K_{iq} (when NADH is a product inhibitor) is consistent with a random mechanism, since for such a mechanism $K_i(\text{slope}) = K_{iq}(1 + B/K_{ib})$ (Cleland, 1970). When ethanol concentration was reduced to 10.8 mM (approximately one-tenth of the K_m for ethanol) and K_i(NADH) was redetermined, it was found to be similar to that obtained from initial velocity (Table IV). The K_{ib} (for ethanol) can also be calculated for this mechanism from the ratio of ϕ coefficients (Table I). However, the all-competitive patterns for NAD–ethanol do not fit the random mechanism exactly, since for a mechanism of this type two competitive and two noncompetitive patterns would be expected due to formation of dead-end ternary complexes. The four competitive patterns suggest that the enzyme form reacting with NAD and ethanol does not form dead-end ternary complexes in contrast to the enzyme form reacting with NADH and acetaldehdye where a dead-end ternary complex enzyme·NAD·acetaldehyde is kinetically detectable (see above). Also interesting is the fact that on the NAD side of the reaction, acetaldehyde functions as a competitive product inhibitor at concentrations far below those at which it functions as a substrate (compare K_i for acetaldehyde with K_m for acetaldehyde), further suggesting that the enzyme isomerization may be responsible for the asymmetry in the mechanism.

Conclusion

On the basis of the above considerations, the enzyme in Figure 1 is represented as existing in two isomeric forms: S and S′. The S form resembles classical horse liver alcohol dehydrogenase in its kinetic mechanism and forms detectable dead-end ternary complexes with products. The S′ form catalyzes the random segment of the reaction, forms a binary complex with acetaldehyde at concentrations far below its substrate concentrations, and forms no detectable dead-end ternary complexes. Since our data are insufficient to pinpoint the enzyme species which isomerizes (it may be a free enzyme or a binary or ternary complex with substrates), the rate equation has been derived on the assumption that ternary complex interconversion is rate limiting, employing the King & Altman (1956) procedure for only three enzyme species: SPQ, SQ, and X [where X includes free S and all S′ enzyme species under the rapid equilibrium assumption (Cha, 1968)]. This equation, however, does not describe the mechanism precisely and more work is necessary to characterize the enzyme species participating in the reaction. Although enzyme isomerizations probably occur in every dehydrogenase-catalyzed reaction, they are fast relative to other steps and, therefore, undetectable kinetically, accounting for nonavailability of a model enzyme for a sequential system involving a rate-limiting isomerization.

The results presented show that the catalytic mechanism of SS isozyme is asymmetrical, compulsory ordered like classical alcohol dehydrogenase with NADH, and most probably random with NAD. If enzyme isomerization is responsible for the asymmetry, the complete cycle must include two isomerizations. A rate-limiting isomerization of the (EE) isozyme·NADH complex was observed by Shore & Gutfreund (1970). SS isozyme differs only slightly from EE isozyme in primary structure, making it likely that some events occurring with EE isozyme might be more easily observable with SS isozyme. The kinetic mechanism of pure EE isozyme does show a slight resemblance to that proposed here for SS isozyme in that it has been shown to be partially random by extensive initial velocity studies (Hanes et al., 1972). Isotope-exchange studies using classical preparations of horse liver alcohol dehydrogenase also indicate randomness in the catalytic mechanism (Silverstein & Boyer, 1964). The kinetic mechanism of yeast alcohol dehydrogenase has been found to be random sequential for ethanol oxidation and compulsory ordered for acetaldehyde reduction (Dickinson & Monger, 1973). Hence, the kinetic mechanism of SS isozyme resembles that of yeast alcohol dehydrogenase in its asymmetry; in the rate-limiting step for ethanol oxidation SS isozyme resembles the EE isozyme.

Sequence and crystallographic studies of alcohol dehydrogenase have revealed differences between EE and SS isozymes in the substrate (as opposed to coenzyme) binding area. Two substitutions likely to affect catalytic properties of SS isozyme were found in position 110 (Phe/Leu) and in position 115 (Asp/Ser) (Brändén et al., 1975). More recent crystallographic studies with EE isozyme have revealed the substrate-binding pocket (Brändén, 1977) which consists of three parts: (1) a hydrophilic bottom where the hydride transfer occurs; (2) a large hydrophobic barrel through which a substrate must enter; (3) a rim where both polar and nonpolar residues are present. Residues 110 and 115 both occur in the hydrophobic barrel region of the substrate-binding pocket. The difference in substrate specificity between EE and SS isozymes has been ascribed to amino acid replacements in the hydrophobic barrel, showing by model-building experiments that large molecules like steroids bind mainly in the hydrophobic barrel. Following the same reasoning, it can be assumed that small substrates like ethanol would largely be confined to the hydride transfer area of the substrate-binding pocket, e.g., in the area where there are no amino acid replacements. Consequently, the differences in the kinetic mechanism between the EE and SS isozymes are not readily explainable in terms of amino acid replacements, unless it is also assumed that the conformation of the coenzyme binding area is altered by amino acid replacements in the substrate-binding site.

Acknowledgments

The authors express their gratitude to Dr. W. W. Cleland of the University of Wisconsin for his highly critical reading of the manuscript and for deriving the rate equation and to Dr. Allen Gold of Columbia University for help in setting Haldanes. We also thank Drs. T. Chase and R. Harvey of

Rutgers University for helpful discussions.

References

Bäcklin, K.-I. (1958) *Acta Chem. Scand. 12*, 1279.
Bonnichsen, R. K., & Wassén, A. M. (1948) *Arch. Biochem. 18*, 361.
Brändén, C.-A. (1977) in *Pyridine Nucleotide-Dependent Dehydrogenases* (Sund, H., Ed.) pp 325–338, de Gruyter, Berlin.
Brändén, C.-I., Jörnvall, H., Eklund, H., & Furugren, B. (1975) *Enzymes, 3rd Ed. 11*, 103–190.
Cha, S. (1968) *J. Biol. Chem. 243*, 820.
Cleland, W. W. (1970) *Enzymes, 3rd Ed. 2*, 1–65.
Dalziel, K. (1957) *Acta Chem. Scand. 11*, 1706.
Dalziel, K. (1963a) *J. Biol. Chem. 238*, 1538.
Dalziel, K. (1963b) *J. Biol. Chem. 238*, 2850.
Dalziel, K. (1975) *Enzymes, 3rd Ed. 11*, 1–60.
Dalziel, K., & Dickinson, F. M. (1966a) *Biochem. J. 100*, 491.
Dalziel, K., & Dickinson, F. M. (1966b) *Biochem. J. 100*, 34.
Dickinson, F. M., & Monger, G. P. (1973) *Biochem. J. 131*, 261.
Dworschack, R. T., & Plapp, B. V. (1977) *Biochemistry 16*, 111.
Eklund, H., Brändén, C.-I., & Jörnvall, H. (1976) *J. Mol. Biol. 102*, 61.
Hanes, C. S., Bronskill, P. M., Gurr, P. A., & Wong, J. T.-F. (1972) *Can. J. Biochem. 50*, 1385.
Jörnvall, H. (1970a) *Eur. J. Biochem. 16*, 25.
Jörnvall, H. (1970b) *Eur. J. Biochem. 16*, 41.
King, E. L., & Altman, C. J. (1956) *J. Phys. Chem. 60*, 1375.
Lineweaver, H., & Burk, D. (1934) *J. Am. Chem. Soc. 58*, 658.
Pietruszko, R., Clark, A., Graves, J. M. H., & Ringold, H. J. (1966) *Biochem. Biophys. Res. Commun. 23*, 526.
Ryzewski, C. N., & Pietruszko, R. (1977) *Arch. Biochem. Biophys. 183, 73.*
Shore, J. D., & Gutfreund, H. (1970) *Biochemistry 9*, 4655.
Silverstein, E., & Boyer, P. D. (1964) *J. Biol. Chem. 239*, 3908.
Sund, H., & Theorell, H. (1963) *Enzymes, 2nd Ed. 7*, 25–83.
Theorell, H., & Chance, B. (1951) *Acta Chem. Scand. 5*, 1127.
Theorell, H., & McKinley-McKee, J. S. (1961) *Acta Chem. Scand. 15*, 1797.
Theorell, H., & Yonetani, T. (1962) *Arch. Biochem. Biophys., Suppl. 1*, 209.
Theorell, H., & Tatemoto, K. (1970) *Acta Chem. Scand. 24*, 3069.
Theorell, H., Taniguchi, S., Åkeson, Å., & Skursky, L. (1966) *Biochem. Biophys. Res. Commun. 24*, 603.
Theorell, H., Åkeson, Å., Liszka-Kopec, B., & de Zalenski, C. (1970) *Arch. Biochem. Biophys. 139*, 241.
Wilkinson, G. N. (1961) *Biochem. J. 80*, 324.
Winer, A. D., & Theorell, H. (1960) *Acta Chem. Scand. 14*, 1729.
Wratten, C. C., & Cleland, W. W. (1963) *Biochemistry 2*, 935.
Wratten, C. C., & Cleland, W. W. (1965) *Biochemistry 4*, 2442.

Isolation and Sequence of the Pyridoxal 5′-Phosphate Active-Site Peptide from *Rhodospirillum rubrum* Ribulose-1,5-bisphosphate Carboxylase/Oxygenase†

Peter D. Robison, William B. Whitman,‡ Florence Waddill, Austen F. Riggs, and F. Robert Tabita*

ABSTRACT: Ribulose-1,5-bisphosphate carboxylase/oxygenase from *Rhodospirillum rubrum* was modified with pyridoxal 5′-phosphate and then reduced with sodium borohydride. Both carboxylase and oxygenase activities were lost when one molecule of pyridoxal 5′-phosphate was bound per enzyme dimer. Peptide maps of modified enzyme showed one N^6-(phosphopyridoxal)lysine-containing peptide. This peptide was isolated by gel filtration and cation-exchange chromatography and its sequence determined as Ala-Leu-Gly-Arg-Pro-Glu-Val-Asp-(PLP-Lys)-Gly-Thr-Leu-Val-Ile-Lys. Since activation of the enzyme with Mg^{2+}/CO_2 enhances pyridoxal 5′-phosphate modification and subsequent inactivation and the substrate ribulose bisphosphate protects against modification, the modified lysyl group is most certainly at the catalytic site and not at the activation site of the enzyme.

Ribulose-1,5-bisphosphate carboxylase/oxygenase is the primary catalyst of photosynthetic carbon fixation and also catalyzes the first step in photorespiratory glycolate production [see Jensen & Bahr (1977) for a review]. The importance of ribulose-1,5-bisphosphate carboxylase/oxygenase in carbon assimilation and crop productivity mandates a complete understanding of the enzyme's chemical structure and mode of action. While the enzyme from higher plants contains eight large (catalytic) and eight small subunits and has a molecular weight of 560 000 (Paulsen & Lane, 1966), a structurally simpler protein which is a dimer of large subunits (M_r 114 000) is easily isolated from the photosynthetic bacterium *Rhodospirillum rubrum* (Tabita & McFadden, 1974a,b).

Considering its importance, little is known about the active-site structure of this enzyme; however, recent evidence

† From the Department of Microbiology (P.D.R., W.B.W., and F.R.T.) and the Department of Zoology (F.W. and A.F.R.), The University of Texas at Austin, Austin, Texas 78712. *Received March 31, 1980.* This work was supported by National Institutes of Health Grant GM-24497 and Robert A. Welch Foundation Grant F-691 to F.R.T., by National Institutes of Health Postdoctoral Fellowship GM-06817 to P.D.R., and by National Institutes of Health Grant GM-21314 to A.F.R.

‡ Present address: Department of Microbiology, University of Illinois, Urbana, IL 61801.

0006-2960/80/0419-4848$01.00/0