Maness, P. F., Perry, M. E., & Levy, B. T. (1983) *J. Biol. Chem. 258*, 4055-4058.
Nishizuka, Y. (1986) *Science 233*, 305-312.
Nishizuka, Y. (1988) *Nature 334*, 661-665.
Ohno, S., Kawasaki, H., Imajoh, S., Suzuki, K., Inagaki, M., Yokokura, H., Sakoh, T., & Hidaka, H. (1987) *Nature 325*, 161-166.
Ono, Y., Fujii, T., Ogita, K., Kikkawa, U., Igarashi, K., & Nishizuka, Y. (1988) *J. Biol. Chem. 263*, 6927-6932.
Rapaport, E., & Zamecnik, P. C. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 3984-3988.
Speizer, L. A., Watson, M. J., Kantnev, J. R., & Brunton, L. L. (1989) *J. Biol. Chem. 264*, 5581-5585.
Takai, Y., Kishimoto, A., Iwasa, Y., Kawahara, Y., Mori, T., & Nishizuka, Y. (1979) *J. Biol. Chem. 254*, 3692-3695.
Tamaoki, T., Nomoto, H., Takahashi, I., Kato, Y., Morimoto, M., & Tomita, F. (1986) *Biochem. Biophys. Res. Commun. 135*, 397-402.
Turner, R. S., Kemp, B. E., & Kuo, J. F. (1985) *J. Biol. Chem. 260*, 11503-11507.
Varshavsky, A. (1983) *J. Theor. Biol. 105*, 707-714.
Walton, G. M., Bertics, P. J., Hudson, L. G., Vedvick, T. S., & Gill, G. N. (1987) *Anal. Biochem. 161*, 425-437.
Whitehouse, S., & Walsh, D. A. (1983) *J. Biol. Chem. 258*, 3682-3692.
Whitehouse, S., Feramisco, J. R., Casnellie, J. E., Krebs, E. G., & Walsh, D. A. (1983) *J. Biol. Chem. 258*, 3693-3701.
Woodgett, J. R., & Hunter, T. (1987) *J. Biol. Chem. 262*, 4836-4843.
Zamecnik, P. C. (1983) *Anal. Biochem. 134*, 1-10.

Steady-State and Pre-Steady-State Kinetics of Propionaldehyde Oxidation by Sheep Liver Cytosolic Aldehyde Dehydrogenase at pH 5.2. Evidence That the Release of NADH Remains Rate-Limiting in the Enzyme Mechanism at Acid pH Values

Jeremy P. Hill,*,‡ Leonard F. Blackwell,‡ Paul D. Buckley,‡ and Rosemary L. Motion§

Department of Chemistry and Biochemistry, Massey University, Palmerston North, New Zealand, and New Zealand Dairy Research Institute, Palmerston North, New Zealand

Received August 2, 1990; Revised Manuscript Received October 15, 1990

ABSTRACT: The k_{cat} value for the oxidation of propionaldehyde by sheep liver cytosolic aldehyde dehydrogenase increased 3-fold, from 0.16 s^{-1} at pH 7.6 to 0.49 s^{-1} at pH 5.2, in parallel with the increase in the rate of displacement of NADH from binary enzyme·NADH complexes. A burst in nucleotide fluorescence was observed at all pH values consistent with the rate of isomerization of binary enzyme·NADH complexes constituting the rate-limiting step in the steady state. No substrate activation by propionaldehyde was observed at pH 5.2, but the enzyme exhibited dissociation/association behavior. The inactive dissociated form of the enzyme was favored by low enzyme concentration, low pH, and low ionic strength. Propionaldehyde protected the enzyme against dissociation.

MacGibbon et al. (1977a,b,c) established that sheep liver cytosolic aldehyde dehydrogenase follows a compulsory order mechanism with NAD^+ as the leading substrate (Scheme I) at pH 7.6, and this was also found to be the case at pH 7.0 (Hart & Dickinson, 1982; Dickinson, 1985). In Scheme I, *E·NADH represents a conformationally rearranged form of the enzyme which must isomerize before NADH can be released from its binding site. Blackwell et al. (1987) have shown that the two-step release of NADH from the enzyme (Scheme I, eq 5) is rate-limiting in the mechanism for the oxidation of propionaldehyde at pH 7.6.

Scheme I

$$E + NAD^+ \rightleftharpoons E\cdot NAD^+ \quad (1)$$

$$E\cdot NAD^+ + \text{aldehyde} \rightleftharpoons E\cdot NAD^+\cdot\text{aldehyde} \quad (2)$$

$$E\cdot NAD^+\cdot\text{aldehyde} \rightleftharpoons E\cdot NADH\cdot\text{acyl} \quad (3)$$

$$E\cdot NADH\cdot\text{acyl} \rightarrow {}^*E\cdot NADH + \text{acid} \quad (4)$$

$$^*E\cdot NADH \rightleftharpoons E\cdot NADH \rightleftharpoons E + NADH \quad (5)$$

At high concentrations of propionaldehyde, a 3-fold activation of the steady-state rate occurs, and this has been explained on the basis of an increase in the rate of NADH release caused by binding of the aldehyde to the enzyme·NADH complexes (Dickinson, 1985). Studies with *trans*-4-(*N*,*N*-dimethylamino)cinnamaldehyde (DACA) (Buckley & Dunn, 1982; Dunn & Buckley, 1985) and with *trans*-cinnamaldehyde (Dickinson & Haywood, 1986) suggest that at acidic pH values acyl-enzyme hydrolysis is slow and thus becomes the rate-limiting step in the mechanism. The limited solubility of both *trans*-cinnamaldehyde and DACA restricts the concentration range over which kinetic measurements can be made with these substrates. No such problems are encountered when propionaldehyde is used as a substrate for the enzyme. The purpose of this study was therefore to investigate the mechanism of sheep liver cytosolic aldehyde dehydrogenase at low pH values using propionaldehyde as a substrate, to determine whether acyl-enzyme hydrolysis is the rate-limiting step in the oxidation for aliphatic aldehydes.

Experimental Procedures

Materials. NADH (grade III) and NAD^+ (grade III) were purchased from Sigma Chemical Co. (St. Louis, MO). Pro-

‡ Massey University.
§ New Zealand Dairy Research Institute.

0006-2960/91/0430-1390$02.50/0

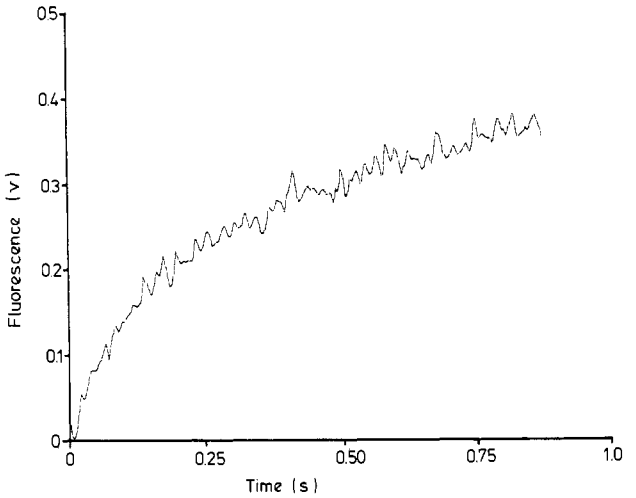

FIGURE 1: Burst in NADH fluorescence at pH 5.0. Enzyme (2.3 μM) and NAD^+ (2 mM) were rapidly mixed with propionaldehyde (20 mM) in constant ionic strength buffer (I = 0.1 M), pH 5.0 (see Methods). The data were fitted as described under Experimental Procedures. The sweep time was 2.0 s and the voltage setting 0.5 V (1.66 μM NADH was equivalent to 1 V).

Table I: Effect of pH on the Pre-Steady-State Fluorescence Burst Rate Constant[a]

pH	rate constant (s^{-1})
5.0	9.9
5.2	9.0
6.0	8.5
7.0	11.5
7.6	14.0

[a] The fluorescence burst rate constant was determined under the conditions described in Figure 1 using constant ionic strength buffers (see Methods) of the appropriate pH.

pionaldehyde was prepared as described by MacGibbon et al. (1977b).

Methods. Sheep liver cytosolic aldehyde dehydrogenase was prepared by the method of MacGibbon et al. (1979) incorporating a pH column purification step (Dickinson et al., 1981). Constant ionic strength buffers (I = 0.1 M) in the pH range 5–7 were prepared according to Dawson et al. (1969) as follows: pH 5.0–5.5 (0.2 M sodium acetate/8.5 M acetic acid/5.0 M NaCl), pH 6.0–7.0 (0.5 M NaH_2PO_4/4.0 M Na_2HPO_4/5.0 M NaCl). Rapid kinetic experiments were performed as described by Blackwell et al. (1987) and MacGibbon et al. (1977a,b) using a Durrum-Gibson D110 stopped-flow instrument. Steady-state kinetic experiments were performed at 25 °C by following the nucleotide absorbance at 340 nm in a Hewlett Packard diode array spectrophotometer. Gel filtration chromatography was performed on a Pharmacia FPLC system using a calibrated Superose 6 HR 10/30 column.

Results and Discussion

A pre-steady-state burst in nucleotide fluorescence (as shown in Figure 1) was observed at all pH values investigated between 5.0 and 7.6 when enzyme was mixed with NAD^+ and propionaldehyde in the stopped-flow apparatus. The magnitude of the rate constants for the burst process as a function of pH is given in Table I and shows only a small decrease upon lowering the pH from 7.6 to 5.0. Similar results are also reported by Dickinson (1986), where the value of the rate constant for the fluorescence burst remained essentially constant between pH 5.5 and 7.0. Hart and Dickinson (1982) found that there was a more rapid burst in NADH absorbance

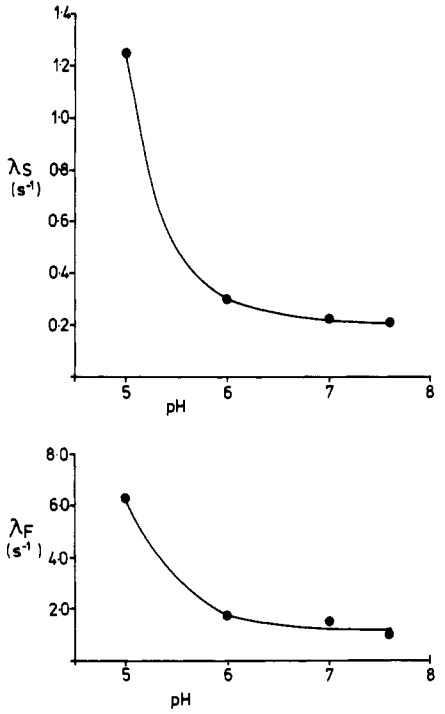

FIGURE 2: Effect of pH on the displacement of NADH. NADH displacement experiments were carried out over the pH range 5–10 using constant ionic strength buffers (I = 0.1 M; see Methods). Solutions containing enzyme (9.0 μM) and NADH (40 μM) in 3.3 mM pH 7.3 phosphate buffer were rapidly mixed with solutions containing NAD^+ (2.0 mM) in the buffer of the appropriate pH.

than in NADH fluorescence upon rapidly mixing sheep liver cytosolic aldehyde dehydrogenase, NAD^+, and propionaldehyde. Dickinson and Haywood (1986) suggest that the absorbance burst represents the rapid formation of a ternary acyl-enzyme·NADH complex in which the presence of the acyl group quenches enzyme-bound NADH fluorescence and that the fluorescence burst represents the relief of fluorescence quenching upon acyl-enzyme hydrolysis to produce a fluorescent binary enzyme·NADH complex. If the pre-steady-state burst process monitored in nucleotide fluorescence is due to hydrolysis of the acyl-enzyme intermediate, then such a step cannot be the rate-determining step in the steady-state turnover of this enzyme. Thus, for propionaldehyde oxidation, the steady-state rate-limiting steps must occur after acyl-enzyme hydrolysis and probably involve enzyme·NADH complexes of Scheme I (eq 5) as at pH 7.6.

Experiments involving displacement of NADH from premixed solutions of enzyme and NADH by NAD^+ were therefore carried out, and a biphasic decay in nucleotide fluorescence was observed at all pH values between 7.6 and 5.0. Thus, as has been shown to be the case at pH 7.6 (Blackwell et al., 1987), the isomerization step (Scheme I, eq 5) is controlling the rate of NADH release at low pH values also. The apparent first-order decay constants for the fast and slow phases of the displacement process (λ_F and λ_S) as a function of pH are shown in Figure 2 where large increases in both λ_F and λ_S are observed upon lowering the pH from 6.0 to 5.0. If NADH release is rate-limiting, then the displacement data suggest that the steady-state rate of oxidation of propionaldehyde at saturating concentrations of substrate should be faster at lower pH values. The question of whether the isomerization step revealed by the displacement experiments lies on the mechanistic pathway and hence controls the rate of propionaldehyde oxidation at pH 5.0 can be decided by a comparison of the steady-state rate of propionaldehyde

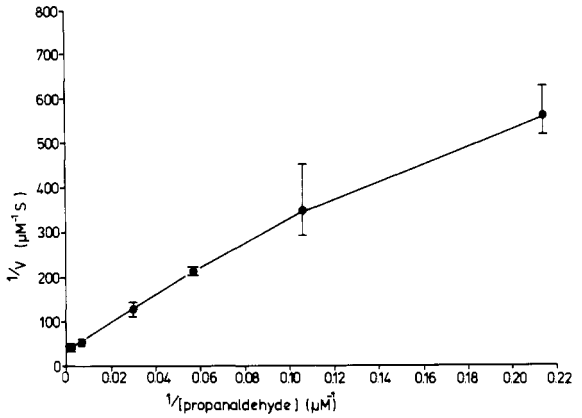

FIGURE 3: Double-reciprocal plot for the oxidation of propionaldehyde by the enzyme at pH 5.2. Enzyme (40 nM) was added last to assays containing 1 mM NAD^+ and propionaldehyde at 25 °C, and the rate of NADH production was followed spectrophotometrically. Points on the plot are derived from six repeat measurements and error bars drawn to show the range of the rates measured at each propionaldehyde concentration.

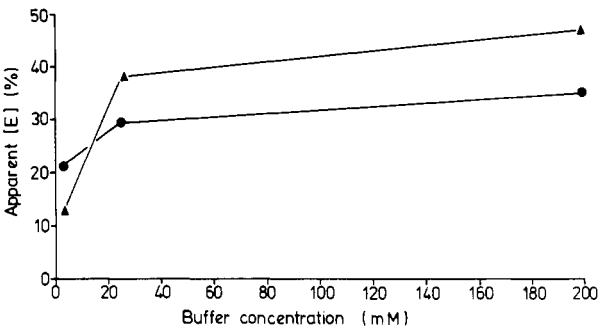

FIGURE 4: Effect of ionic strength and pH on the rate of propionaldehyde oxidation. Enzyme (73 nM as determined by adding a high enzyme concentration of 3 μM last to an assay containing 20 mM propionaldehyde and 1 mM NAD^+ in 25 mM phosphate buffer, pH 7.6; see Methods) was preincubated for 5 min in 2.5, 25, and 200 mM sodium phosphate buffer, pH 7.6 (▲), or in 2.5, 25, and 200 mM sodium acetate buffer, pH 5.2 (●), containing 1 mM NAD^+ followed by the addition of 6 mM propionaldehyde, and the production of NADH was followed spectrophotometrically.

oxidation and the rate of isomerization of the binary enzyme·NADH complexes.

The substrate dependence of the steady-state rate of oxidation of propionaldehyde by the enzyme at pH 5.2 was determined and is shown in Figure 3. The interesting feature of the double-reciprocal plot of the data is the nonlinearity at low aldehyde concentrations (<50 μM) but linear dependence of the oxidation rates over the higher aldehyde concentration range (up to 20 mM), which is the reverse of the behavior at pH 7.6. From the intercept of the linear section of the plot (>50 μM propionaldehyde) with the $1/v$ axis, a k_{cat} value of 0.49 s^{-1} was determined which is approximately 3 times faster than the steady-state rate at pH 7.6 (Blackwell et al., 1987). Further increases in the NAD^+ concentration above 1 mM, as used in Figure 2, had a negligible effect on the k_{cat} value.

The k_{cat} value for the rate of oxidation can also be calculated for the mechanism shown in Scheme I by assuming that the isomerization step (eq 5) lies on the pathway if the rate constants for the NADH release steps are known. It is difficult to extract the individual rate constants from λ_S and λ_F which are complex functions of these rate constants (MacGibbon et al., 1977a,b,c); however, the ratio of the product of λ_F and λ_S to the sum of λ_F and λ_S takes the same form as the King–Altman expression for k_{cat} derived for Scheme I (Segel, 1975). Hence

$$k_{cat} = \lambda_S\lambda_F/(\lambda_S + \lambda_F) \quad (6)$$

Substitution of the λ_S and λ_F values determined at pH 5.2 from the NADH displacement experiments into eq 1 gives a value for k_{cat} (0.52 s^{-1}) which is the same within experimental error as the value (0.49 s^{-1}) determined from steady-state measurements at saturating propionaldehyde concentration. Thus, we conclude that the NADH release steps lie on the reaction pathway and are entirely rate-limiting at pH 5.2 for propionaldehyde, as previously demonstrated at pH 7.6 (Blackwell et al., 1987).

Independent confirmation of this conclusion was provided by the inhibition of the steady-state rate of oxidation of propionaldehyde by magnesium chloride at pH 5.2. Magnesium chloride inhibits sheep liver cytosolic aldehyde dehydrogenase throughout the pH range 6–8 (Bennett et al., 1983) which Dickinson and Hart (1982) suggest is due to the slower release of NADH from the E·NADH·Mg^{2+} complex compared with NADH release from the E·NADH complex. The direct observation that the displacement rate constants are decreased in the presence of magnesium ions (Bennett et al., 1983) supports this explanation. Hence, the observation in this study of 85% inhibition of propionaldehyde (20 mM) oxidation by 3.5 mM magnesium chloride under the conditions described in Figure 3 is strong supporting evidence that NADH release is indeed rate-limiting at acidic pH values.

The slight curvature of the Lineweaver–Burk plot at pH 5.2 at low concentrations of propionaldehyde (<50 μM) shown in Figure 3 (toward the 1/[propionaldehyde] axis) could be interpreted in terms of a propionaldehyde-induced activation of NADH release from the enzyme as proposed by Dickinson (1985) at pH 7.0. However, this appears unlikely since if such activation was occurring the k_{cat} value calculated from the λ_S and λ_F values and that determined from the steady-state kinetic measurements would not agree since propionaldehyde is not present in the NADH displacement experiments. The lack of activation of the steady-state rate by 20 mM propionaldehyde at pH 5.2 finds its explanation in the pH dependence of the K_m value for propionaldehyde which was determined from the linear region in Figure 3 to be 58 μM. This represents a substantial increase over the K_m value of approximately 2.0 μM determined above pH 7.0 (MacGibbon et al., 1977c; Hart & Dickinson, 1982). Thus, the binding of propionaldehyde to the site on the enzyme in which it is oxidized is significantly weaker at lower pH values, and hence much higher concentrations of propionaldehyde are required to attain saturation. This explanation is supported by the findings of Dickinson (1986), where a group on the enzyme with a pK_a value of approximately 6.6 was proposed to affect aldehyde binding such that the K_m value was increased at low pH.

The origin of this curvature may be found in the observation that predilution of the enzyme at pH 7.6 prior to the addition of propionaldehyde gives lower aldehyde oxidation rates than those found in assays where the enzyme has not been prediluted prior to aldehyde addition (Blackwell et al., 1987). Propionaldehyde protects against the inactivation caused by predilution. Incubation of the enzyme with 200 μM propionaldehyde in 25 mM phosphate buffer, pH 7.6, for 5 min gives a higher aldehyde oxidation rate (approximately 2-fold), upon the addition of NAD^+, than preincubation of the enzyme and NAD^+ for a similar period of time prior to the addition of aldehyde.

In the present study at pH 5.2, in 25 mM sodium acetate buffer, a similar phenomenon was observed (Figure 4).

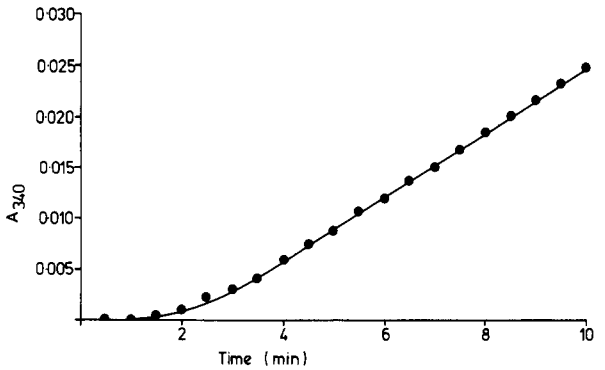

FIGURE 5: Effect of prolonged enzyme preincubation on the steady-state oxidation of propionaldehyde in 2.5 mM sodium acetate buffer, pH 5.2. Enzyme (7.6 nM) was preincubated in 2.5 mM sodium acetate buffer, pH 5.2, containing 1 mM NAD^+ for 30 min prior to the addition of 6 mM propionaldehyde.

Predilution of the enzyme for 15 min prior to the addition of 20 mM propionaldehyde caused appreciable inactivation of the enzyme at both pH 7.6 and pH 5.2. As shown in Figure 4, the extent of the inactivation was greater at lower ionic strengths at both pH values and in pH 5.2 25 and 200 mM sodium acetate buffers. When enzyme (0.07 μM) was prediluted in 2.5 mM sodium acetate buffer for 0.5 h prior to the addition of propionaldehyde (6 mM), there was essentially no oxidation of aldehyde for approximately 90 s after which the oxidation of aldehyde increased slowly over a 3-min period, the trace finally becoming linear (Figure 5). When the above experiment was repeated at pH 7.6, no detectable oxidation of aldehyde occurred over the 10-min assay period.

Dilution of polymeric proteins should cause some dissociation to lower molecular weight components (Klotz et al., 1975), although for many proteins the concentration at which such dissociations occur is so low as not to interfere with their experimental study or use. Klotz et al. (1975) also list a number of proteins in which the binding of ligands promotes association. The binding of ligand to the associated form of the protein shifts the equilibrium to the left as in Scheme II, thus explaining the protective effect of propionaldehyde.

Scheme II

$$\text{associated protein·ligand} \underset{+\text{ligand}}{\overset{-\text{ligand}}{\rightleftharpoons}} \text{associated protein} \rightleftharpoons \text{dissociated protein}$$

Upon gel filtration chromatography of sheep liver cytosolic aldehyde dehydrogenase (see Methods) at high enzyme concentrations (44 μM) and at pH 7.6 in 25 mM sodium phosphate buffer, one major active peak (M_r 200000, 99% of eluted protein) and one minor inactive peak (M_r 50000–100000, 1% of eluted protein) eluted from the Superose 6 column (Figure 6a). At pH 5.0 and in 25 mM sodium acetate buffer, the active peak corresponded to 95% and the nonactive peak to 5% of the eluted protein (Figure 6b). These ratios changed upon lowering the preload enzyme concentration 10-fold to 88% (active) and 12% (inactive) at pH 7.6 (Figure 6c) and to 52% (active) and 48% (inactive) at pH 5.0 (Figure 6d). Attempts to restore enzyme activity upon concentration of inactive protein samples by ultrafiltration were unsuccessful. Either the enzyme undergoes a nonreversible inactivation upon dissociation (however, the results shown in Figure 5 suggest that this is not the case) or the reassociation process is slow in the absence of an association-promoting ligand such as propionaldehyde. Dilution has therefore induced the enzyme to dissociate from a tetramer into an inactive lower molecular

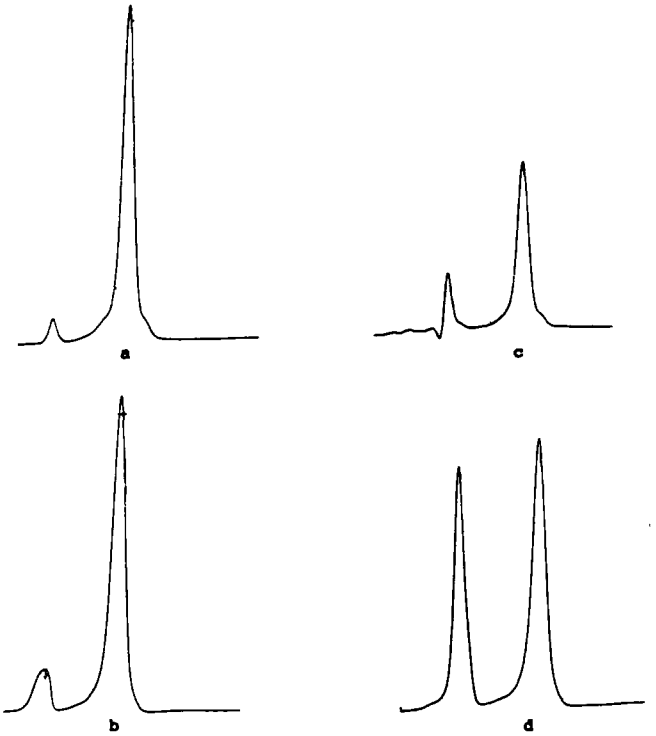

FIGURE 6: Gel filtration chromatography of the enzyme. Enzyme was prediluted for 15 min prior to being loaded (150-μl samples) onto a Superose 6HR 10/30 gel filtration column (flow rate 0.5 mL min^{-1}). (a) 44 μM enzyme in 25 mM sodium phosphate buffer, pH 7.6. (b) 44 μM enzyme in 25 mM sodium acetate buffer, pH 5.0. (c) 4.4 μM enzyme in 25 mM sodium phosphate buffer, pH 7.6. (d) 4.4 μM enzyme in 25 mM sodium acetate buffer, pH 5.0. Sample peaks were collected in approximately 2-mL volumes off the column. These were then assayed spectrophotometrically upon adding propionaldehyde (20 mM) and NAD^+ (1 mM).

weight form. [It is unlikely that a regulatory protein component dissociates upon dilution of this enzyme as mammalian cytosolic aldehyde dehydrogenase are homotetramers (M_r ~200000) formed through the noncovalent binding together of four identical subunits (M_r ~50000).] Thus, sheep liver cytosolic aldehyde dehydrogenase dissociates under conditions of low pH, low ionic strength, and low protein concentration. It would appear that ligand binding (in this case propionaldehyde) favors the associated active form of the enzyme (Scheme II). The nonlinearity in the Lineweaver–Burk plot at pH 5.2 (Figure 3) can therefore be best explained in terms of an increase in the amount of associated enzyme as the concentration of propionaldehyde is increased. Once the enzyme is completely associated (above 50 μM concentrations of propionaldehyde), the relationship between propionaldehyde concentration and oxidation rate is linear (Figure 3).

In conclusion, the oxidation of propionaldehyde by sheep liver cytosolic aldehyde dehydrogenase is limited by the release of NADH from the enzyme at pH values below 7.0. This finding is different from the conclusions drawn from studies using DACA where acyl-enzyme hydrolysis was proposed to be rate-determining in the oxidation of this aldehyde by the enzyme at low pH values (Buckley & Dunn, 1982; Dunn & Buckley, 1985). Caution should therefore be applied when comparing kinetic results from studies using structurally different aldehydes such as DACA and propionaldehyde, particularly in view of the possibility of substrate-induced association/dissociation phenomena. Due to the close sequence homology between all liver cytosolic aldehyde dehydrogenases (Hempel & Jörnvall, 1987), it will be interesting to see whether these related enzymes exhibit association/dissociation behavior

and if so the effect of this on kinetic measurements. Mitochondrial forms of the enzyme have been shown to increase in activity upon dissociation (Dickinson & Allanson, 1985; Weiner & Takahashi, 1985).

Registry No. NADH, 58-68-4; propionaldehyde, 123-38-6; aldehyde dehydrogenase, 9028-86-8.

REFERENCES

Bennett, A. F., Buckley, P. D., & Blackwell, L. F. (1983) *Biochemistry 22*, 776-784.

Blackwell, L. F., Bennett, A. F., & Buckley, P. D. (1983) *Biochemistry 22*, 3784-3791.

Blackwell, L. F., Motion, R. L., MacGibbon, A. K. H., Hardman, M. J., & Buckley, P. D. (1987) *Biochem. J. 242*, 803-808.

Buckley, P. D., & Dunn, M. F. (1982) *Prog. Clin. Biol. Res. 114*, 23-35.

Dawson, R. M. C., Elliot, D. C., Elliot, W. H., & Jones, K. M. (1969) *Data for Biochemical Research*, pp 483-500, Oxford University Press, New York.

Dickinson, F. M. (1985) *Biochem. J. 225*, 159-165.

Dickinson, F. M. (1986) *Biochem. J. 238*, 75-82.

Dickinson, F. M., & Hart, G. J. (1982) *Biochem. J. 205*, 443-448.

Dickinson, F. M., & Allanson, S. (1985) *Prog. Clin. Biol. Res. 174*, 71-82.

Dickinson, F. M., & Haywood, G. W. (1986) *Biochem. J. 233*, 877-883.

Dickinson, F. M., Hart, G. J., & Kitson, T. M. (1981) *Biochem. J. 199*, 573-579.

Dunn, M. F., & Buckley, P. D. (1985) *Prog. Clin. Biol. Res. 174*, 15-27.

Hart, G. J., & Dickinson, F. M. (1982) *Biochem. J. 203*, 617-627.

Hempel, J., & Jörnvall, H. (1987) *Prog. Clin. Biol. Res. 232*, 1-14.

Klotz, I. M., Darnall, D. W., & Langerman, N. R. (1975) *Proteins (3rd Ed.) 1*, 293-411.

MacGibbon, A. K. H., Buckley, P. D., & Blackwell, L. F. (1977a) *Biochem. J. 165*, 455-462.

MacGibbon, A. K. H., Blackwell, L. F., & Buckley, P. D. (1977b) *Biochem. J. 167*, 469-477.

MacGibbon, A. K. H., Blackwell, L. F., & Buckley, P. D. (1977c) *Eur. J. Biochem. 77*, 93-100.

MacGibbon, A. K. H., Motion, R. L., Crow, K. E., Buckley, P. D., & Blackwell, L. F. (1979) *Eur. J. Biochem. 96*, 585-595.

Segel, I. H. (1975) *Enzyme Kinetics. Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems*, pp 506-523, Wiley, New York.

Weiner, H., & Takahashi, K. (1985) *Prog. Clin. Biol. Res. 174*, 83-90.

Cooperativity of Papain–Substrate Interaction Energies in the S_2 to S_2' Subsites†

Paul J. Berti,‡,§ Carlos H. Faerman,‡ and Andrew C. Storer*,‡

Protein Engineering Section, Biotechnology Research Institute, 6100 avenue Royalmount, Montréal, Québec, H4P 2R2, Canada, and Department of Biochemistry, McGill University, 3655 Drummond Street, Montréal, Québec, H3G 1Y6, Canada

Received August 21, 1990; Revised Manuscript Received October 17, 1990

ABSTRACT: Enzyme–substrate contacts in the hydrolysis of ester substrates by the cysteine protease papain were investigated by systematically altering backbone hydrogen-bonding and side-chain hydrophobic contacts in the substrate and determining each substrate's kinetic constants. The observed specificity energies [defined as $\Delta\Delta G_{obs} = -RT \ln [(k_{cat}/K_M)_{first}/(k_{cat}/K_M)_{second})]$] of the substrate backbone hydrogen bonds were −2.7 kcal/mol for the P_2 NH and −2.6 kcal/mol for the P_1 NH when compared against substrates containing esters at those sites. The observed binding energies were −4.0 kcal/mol for the P_2 Phe side chain, −1.0 kcal/mol for the P_1' C=O, and −2.3 kcal/mol for the P_2' NH. The latter three values probably all significantly underestimate the incremental binding energies. The P_2 NH, P_2 Phe side-chain, and P_1 NH contacts display a strong interdependence, or cooperativity, of interaction energies that is characteristic of enzyme–substrate interactions. This interdependence arises largely from the entropic cost of forming the enzyme–substrate transition state. As favorable contacts are added successively to a substrate, the entropic penalty associated with each decreases and the free energy expressed approaches the incremental interaction energy. This is the first report of a graded cooperative effect. Elucidation of favorable enzyme–substrate contacts remote from the catalytic site will assist in the design of highly specific cysteine protease inhibitors.

Papain (EC 3.4.22.3) is the most studied and consequently best understood member of the papain superfamily of cysteine proteases. The hydrolytic mechanism is minimally represented kinetically by a three-step process:

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} E{\cdot}S \xrightarrow{k_{+2}} EA + Prod1 \xrightarrow{k_{+3}} E + Prod2$$

The free enzyme and substrate (E + S) associate to form a Michaelis complex (E·S) with a dissociation constant K_S (= k_{-1}/k_{+1}). The carbonyl carbon of the scissile ester or amide bond undergoes nucleophilic attack from the active-site cysteine (Cys 25), forming the covalent acyl–enzyme intermediate (EA) and releasing the alcohol or amine product (Prod1) with

† NRCC Publication No. 32396.
‡ Biotechnology Research Institute.
§ McGill University.

0006-2960/91/0430-1394$02.50/0