Komatsu, S. K., and Feeney, R. E. (1967), *Biochemistry 6,* 1136–1141.
Lange, L. G., Riordan, J. F., and Vallee, B. L. (1974), *Biochemistry 13,* 4361–4370.
Line, W. F., Grohlich, D., and Bezkorovainy, A. (1967), *Biochemistry 6,* 3393–3402.
Marcel, Y. L., Christiansen, K., and Holman, R. T. (1968), *Biochim. Biophys. Acta 164,* 25–34.
Marcus, F., Schuster, S. M., and Lardy, H. A. (1976), *J. Biol. Chem. 251,* 1775–1780.
Markland, F. S., Bacharach, A. D. E., Weber, B. H., O'Grady, T. C., Saunders, G. C., and Omemura, N. (1975), *J. Biol. Chem. 250,* 1301–1310.
Oshino, N., and Sato, R. (1971), *J. Biochem. (Tokyo) 69,* 169–180.
Oshino, N., Imai, Y., and Sato, R. (1966), *Biochim. Biophys. Acta 128,* 13–28.
Ozols, J., and Strittmatter, P. (1966), *J. Biol. Chem. 241,* 4793–4797.
Patthy, L., and Smith, E. L. (1975a) *J. Biol. Chem. 250,* 557–564.
Patthy, L., and Smith, E. L. (1975b) *J. Biol. Chem. 250,* 565–569.
Powers, S. G., and Riordan, J. F. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 2616–2620.
Riordan, J. F. (1973), *Biochemistry 12,* 3915–3923.
Riordan, J. F., Wacker, W. E. C., and Vallee, B. L. (1965), *Biochemistry 4,* 1758–1765.
Riordan, J. F., McElvany, K. D., and Borders, C. L., Jr. (1977), *Science 195,* 884–885.
Rogers, M. J., and Strittmatter, P. (1973), *J. Biol. Chem. 248,* 800–806.
Rogers, M. J., and Strittmatter, P. (1974), *J. Biol. Chem. 249,* 5565–5560.
Schmidt, E., and Fischer, H. (1920), *Ber. Dtsch. Chem. Ges. 53,* 1529.
Shimakata, T., Mihara, K., and Sato, R. (1972) *J. Biochem. (Tokyo) 72,* 1163–1174.
Sokolovsky, M., Riordan, J. F., and Vallee, B. L. (1966), *Biochemistry 5,* 3582–3589.
Sokolovsky, M., Harell, D., and Riordan, J. F. (1969), *Biochemistry 8,* 4140–4145.
Spatz, L., and Strittmatter, P. (1973), *J. Biol. Chem. 248,* 793–799.
Strittmatter, P. (1971), *J. Biol. Chem. 246,* 1017–1024.
Strittmatter, P., and Enoch, H. G. (1978), *Methods Enzymol. 52,* 188–193.
Strittmatter, P., Spatz, L., Corcoran, D., Rogers, M. J., Setlow, B., and Redline, R. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 4565–4569.
Strittmatter, P., Enoch, H. G., and Fleming, P. (1978), *Methods Enzymol. 52,* 206–211.
Strittmatter, P., Fleming, P., Connors, M., and Corcoran, D. (1978), *Methods Enzymol. 52,* 97–101.
Tsao, D., Azari, P., and Phillips, J. L. (1974), *Biochemistry 13,* 408–413.
Wolff, J., Alazard, R., Camier, M., Griffin, J. H., and Cohen, P. (1975), *J. Biol. Chem. 250,* 5215–5220.
Yazawa, M., and Noda, L. H. (1976), *J. Biol. Chem. 251,* 3021–3026.

Kinetic Study on the Reaction Mechanism of Pantothenase: Existence of an Acyl-Enzyme Intermediate and Role of General Acid Catalysis†

R. Kalervo Airas

ABSTRACT: A kinetic study was performed on the reaction mechanism of pantothenase (EC 3.5.1.22) catalyzed hydrolysis of the pantothenic acid. A nonlinear progress curve is derived if the reaction occurs at low buffer concentrations. The nonlinearity is due to partial reversibility of the reaction; an acylenzyme (pantoyl-enzyme) is formed during the reaction, and β-alanine, the other end product, is able to react with the acyl-enzyme and return back to pantothenate. The dependence of the β-alanine return reaction on buffer concentration and on pH suggests a general acid catalysis during the reaction. A reaction mechanism is suggested, in which the NH_3^+ form of β-alanine participates in the return reaction, and the deacylation of the acyl-enzyme is acid catalyzed.

Pantothenase (EC 3.5.1.22) decomposes pantothenic acid into pantoic acid and β-alanine:

$$HOCH_2C(CH_3)_2CH(OH)CONHCH_2CH_2COOH \rightarrow HOCH_2C(CH_3)_2CH(OH)COOH + NH_2CH_2CH_2COOH$$

Previous kinetic studies of the enzyme have shown the existence of an ionizable group with pK = 7.0 at the substrate binding site in the enzyme. The ionizable group affects substrate binding (K_m) but not V_{max}, and the substrate is bound to the basic form of the ionizable group. The pK value and its dependence on temperature ($\Delta H = -50$ kJ/mol) suggest that the ionizable group is a histidine imidazole. The decrease in the activity below pH 7 is due to this imidazole. An anomalous, buffer-dependent fall in the pH-activity curve is found above pH 7, and this fall originally caused the further kinetic studies described in the present paper. The fall was found to be due to nonlinearity of the reaction velocity curve, and, further, the nonlinearity is caused by a partial reverse reaction. A scheme of the reaction mechanism is suggested based on kinetic studies on the dependence of the reaction on the pH, buffer concentration, and the buffering substance.

† From the Department of Biochemistry, University of Turku, SF-20500 Turku 50, Finland. *Received May 24, 1978.*

0006-2960/78/0417-4932$01.00/0 © 1978 American Chemical Society

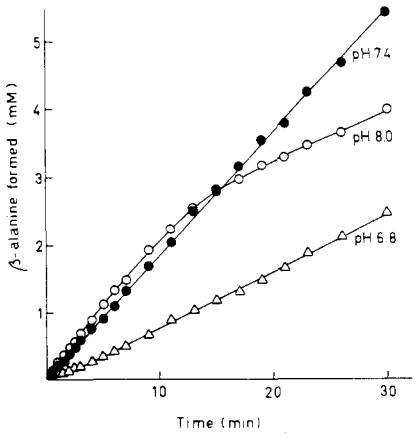


FIGURE 1: Progress curves of the pantothenase catalyzed hydrolysis of pantothenic acid at various pHs. The buffer was 20 mM potassium phosphate and the pantothenate concentration was 30 mM.

Materials and Methods

Materials. Pantothenase was purified from *Pseudomonas fluorescens* UK-1 with a method described previously (Airas et al., 1976). The specific activity of the enzyme preparation was about 120 nkat/mg.

The buffer substances, Mops[1] [3-(*N*-morpholino)propanesulfonic acid], Hepes (*N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid), Tes [*N*-tris(hydroxymethyl)methylaminoethanesulfonicacid], Tricine[*N*-tris(hydroxymethyl)-methylglycine], and Bicine [*N,N*-bis(2-hydroxyethyl)glycine], were obtained from Serva, Heidelberg, West Germany. D-[1-^{14}C]Pantothenate was obtained from NEN Chemicals GmbH, D6072 Dreieichenhain, West Germany. The radioactive β-alanine was prepared by acid hydrolysis of the radioactive pantothenate. The radioactive pantothenate was allowed to stand in 5 M HCl at 60 °C for 20 h. Then the solution was evaporated to dryness, water was added to give about 2 × 10^7 cpm/mL, and the pH was adjusted to 7 with KOH.

Pantothenase Assay. The pantothenase activity was assayed as described previously (Airas, 1976). In the determinations of the progress curves the reaction mixture, 750 μL by total volume, contained 150 μL of each: 150 mM D-pantothenate potassium salt, solution of D-[1-^{14}C]pantothenate, 0.15 μCi/150 μL, buffer (mentioned separately), water or "added compound solution", and the enzyme in 5 mM K_2SO_4. Samples for [^{14}C]-β-alanine determination were taken from the reaction mixture in short intervals and pipetted onto Whatman no. 1 chromatography paper as in normal pantothenase assays (Airas, 1976). The substrate concentration in the reaction mixture was 30 mM. The K_m value ranges from 8 to 16 mM at the pH values used in the measurements, and thus the enzyme was 65–80% saturated with the substrate.

Assay of the β-Alanine Return Reaction. The rate of return of β-alanine into pantothenate was measured with a similar reaction mixture as the pantothenase activity except that the radioactive β-alanine was substituted for the radioactive pantothenate, and a 10 mM concentration of nonradioactive β-alanine was added. The radioactivities of the pantothenate spots (R_f = 0.85) on the chromatograms were counted.

[1] Abbreviations used: Mops, 3-(*N*-morpholino)propanesulfonic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid; Tes, *N*-tris(hydroxymethyl)methylaminoethanesulfonic acid; tricine, *N*-tris(hydroxymethyl)methylglycine; Bicine, *N,N*-bis(2-hydroxyethyl)-glycine.

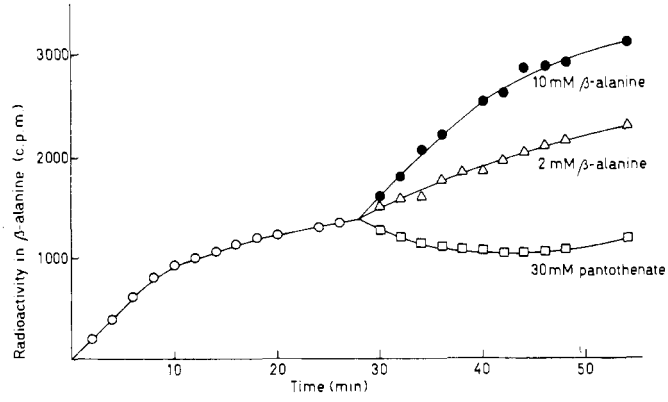


FIGURE 2: Effects of added β-alanine and pantothenate on the reaction velocity curve. At the beginning the reaction mixture (4 × 150 μL) consisted of the pantothenate (37.5 mM), the [1-^{14}C]pantothenate, the buffer (25 mM potassium phosphate pH 8), and the enzyme. The sample volume was 20 μL. At 28 min, β-alanine or pantothenate was added to give the following concentrations: (●) 10 mM β-alanine; (△) 2 mM β-alanine; (□) added 30 mM (total 60 mM) pantothenate. The buffer concentration was thereafter 20 mM, and the sample volume 25 μL. The radioactivity of the β-alanine in the 20 or 25 μL sample is expressed.

Results

Nonlinearity of the Progress Curve. The pantothenase activity was measured as the rate of release of [^{14}C]-β-alanine from [^{14}C]pantothenic acid. The progress of the reaction was followed by taking in short intervals samples for [^{14}C]-β-alanine determination from the same reaction mixture. Examples of progress curves thus derived are presented in Figure 1. In phosphate buffer at pH 6.8–7.4 the curve is essentially a direct line, but at pH 8 it is curved. The nonlinear progress curves include in most cases a nearly linear part at the beginning, then a curved part, and thereafter an only slightly curving part. The strongly curved part of the progress curve falls normally to quite the same product concentration (2–3.5 mM β-alanine). This is true when changes in enzyme concentrations (3–15 nkat/mL), substrate concentrations (5–60 mM), or temperatures (10–30 °C) are considered.

The β-Alanine Return Reaction. If β-alanine is added to the reaction mixture, the progress curve is much longer and nearly linear. The velocity of the release of the radioactive β-alanine is increased again, when cold β-alanine is added after the curving stage (Figure 2). However, the previously formed radioactive β-alanine diminishes in the reaction mixture, if nonradioactive pantothenate is added after the curving stage (or the specific activity of pantothenate is lowered, Figure 2). Added compounds with no or very little effect include pantoate (the other end product), oxalate (inhibitor), glycine, or γ-aminobutyrate (structural relatives to β-alanine). The effects of β-alanine and nonradioactive pantothenate suggest a disappearance of β-alanine from the reaction mixture. This disappearance, however, is not connected with any formation of ammonia nor does it cause any decrease in total radioactivity of the reaction mixture, and, further, no new radioactive spots are found in chromatograms of the reaction mixture. So it is apparent that β-alanine is converted back to pantothenate.

Figure 3 shows direct measurements of the reverse reaction from β-alanine to pantothenate. Radioactive β-alanine but no radioactive pantothenate was added to the reaction mixture. In the presence of substrate the radioactivity from β-alanine is rapidly transferred to pantothenate. If pantothenate is lacking, no reverse reaction can be detected. Even in the presence of pantoate, but no pantothenate, any formation of radioactive pantothenate cannot be detected at least in this

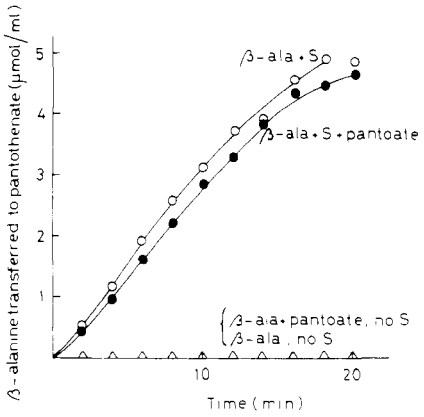


FIGURE 3: The return reaction of β-alanine. The reaction mixture (750 μL) always contained 10 mM β-alanine, 0.15 μCi of the radioactive β-alanine, 20 mM potassium phosphate (pH 8.0), and the enzyme, and in addition the following compounds: (○) 30 mM potassium pantothenate (S); (●) 30 mM potassium pantothenate and 5 mM potassium D-pantoate; (△) no added compounds or 5 mM potassium D-pantoate.

measurement system. These results suggest the following reaction mechanism (in its most simplified form):

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} EP \xrightarrow{k_{+3}} E + P \qquad (1)$$

B

An acyl-enzyme (EP) is formed in the course of the reaction, and the irreversible stage is the hydrolysis of the acyl-enzyme. The liberation of β-alanine (B) is a reversible stage. According to eq 1 a steady-state rate equation can be derived for the enzymatic reaction:

$$v = \frac{V}{c_1 B + c_2} \qquad (2)$$

where

$$V = k_{+3} e$$

$$c_1 = (k_{-1}k_{-2} + k_{+1}Sk_{-2})/(k_{+1}Sk_{+2})$$

$$c_2 = (k_{-1}k_{+3} + k_{+2}k_{+3} + k_{+1}Sk_{+3} + k_{+1}Sk_{+2})/(k_{+1}Sk_{+2})$$

e = total enzyme concentration

The expression of β-alanine concentration can be formed by integration of eq 2:

$$dB = v\,dt = \frac{V}{c_1 B + c_2}\,dt$$

$$\int (c_1 B + c_2)\,dB = \int V\,dt$$

$$\frac{c_1}{2} B^2 + c_2 B = Vt \qquad (3)$$

Equation 3 means a parabolic dependence between reaction time and concentration of formed β-alanine.

Table I shows the velocities of the β-alanine return reaction and the decrease in the rate of the pantothenate hydrolysis. The numbers are essentially equal. In a system of eq 1, this equality means that the rate-limiting step must be at the acylation. Otherwise there would not exist any competition for the acyl-enzyme between the deacylation and the β-alanine return reaction.

Effect of Buffer on the Progress Curve. According to Figure 1 the pH affects the progress curve very strongly in phosphate buffer (pK = 7). In Tes buffer (pK = 7.5) the effect is also quite clear (Figure 4), but in Tris buffer (pK = 8.3) it is much smaller (Figure 5). So between pH 7 and 8.5 the pH effect on the progress curve seems to be dependent on the pK value of the buffer used.

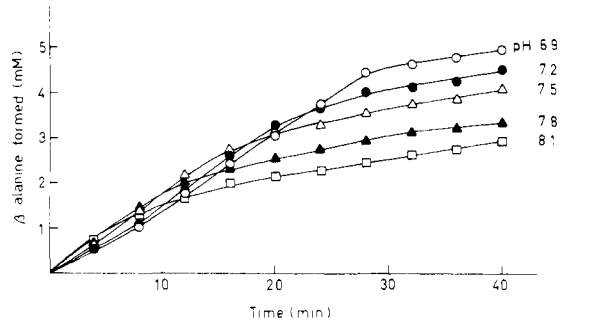


FIGURE 4: Effect of pH of Tes buffer on the reaction velocity curve. The buffer concentration was 5 mM and the pHs were adjusted with KOH. The other conditions of the reactions were as described in the "Materials and Methods".

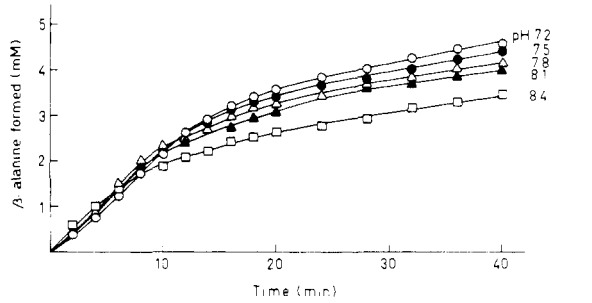


FIGURE 5: Effect of pH of Tris buffer on the reaction velocity curve. The buffer concentration was 3 mM. The pHs were adjusted with H_2SO_4.

TABLE I: Rate of the β-Alanine Return Reaction Compared to the Decrease in the Hydrolysis of the Substrate at Various Buffer Concentrations.[a]

buffer concn (mM)	β-alanine concn[b] (mM)	β-alanine return reaction (μmol/min)	decrease of forward reaction[c] (μmol/min)
1	2.8	0.185	0.173
3.3	4.0	0.172	0.158
5	4.3	0.142	0.149
10	5.0	0.120	0.106
20	5.8	0.091	0.091

[a] The experiment was carried out as in Figure 8. The buffer was Tris-H_2SO_4 (pH 8) and the substrate concentration was 30 mM. β-Alanine (2 mM) was present at the beginning of the β-alanine return measurement. [b] The release and return reactions of β-alanine were measured at the same β-alanine concentration. The increase in β-alanine concentration during the return reaction was taken into account. [c] Initial velocity (0.216 μmol/min) minus the velocity at the given β-alanine concentration.

The backward reaction from β-alanine to pantothenate is also affected by pH when determined in phosphate or Tes buffer (Table II), but in Tris buffer the pH effect is small.

Figure 6a shows the pH effect on the progress curve when various buffers and pH values, pH = pK, are used. The concentration of the acidic form (HA) of the buffers (as well as of the basic form, A^-) remains equal, and [HA] = [A^-]. The curves are essentially similar. Also the velocity of the reverse reaction is equal after a delay at the most acidic pHs (Figure 6b). The results in Figure 6 thus do not show any real dependence of velocity on pH.

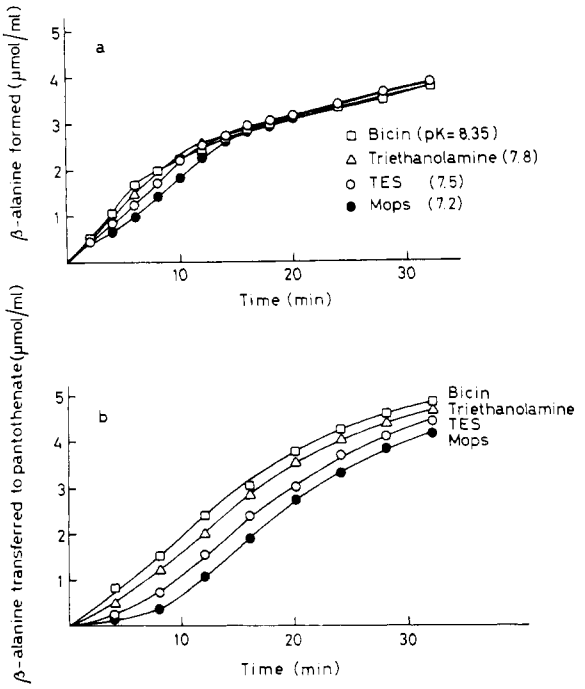


FIGURE 6: Effect of pH on the forward reaction of pantothenate hydrolysis by pantothenase (a), and on the β-alanine return reaction (b) in various buffers when pH = pK. The buffers were 3 mM Mops (KOH), pH 7.2, 3 mM Tes (KOH), pH 7.5, 3 mM triethanolamine (H_2SO_4), pH 7.8, and 3 mM Bicine (H_2SO_4), pH 8.35.

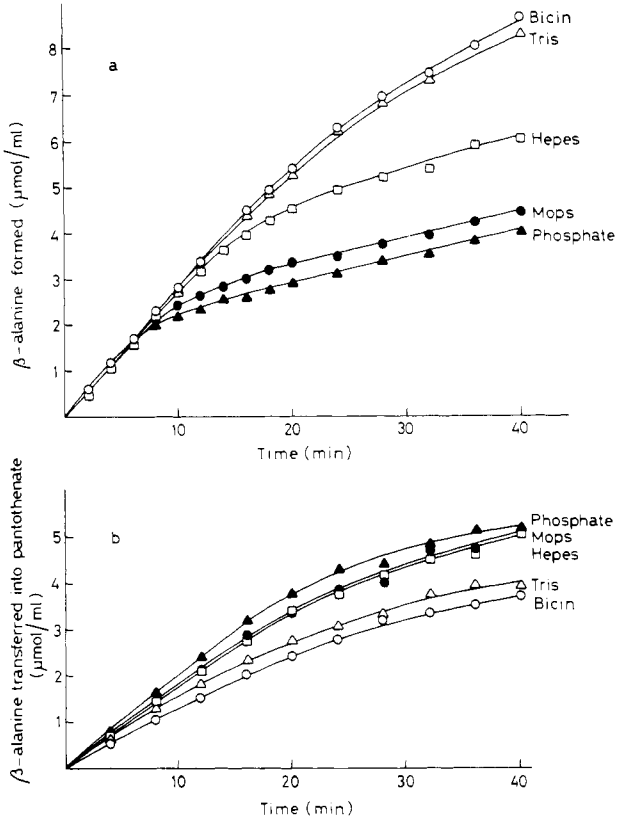


FIGURE 7: Effect of different buffers on the forward reaction of pantothenase (a) and on the return reaction of β-alanine (b). The buffers were 20 mM and the pH 8.0 was adjusted either with H_2SO_4 or with KOH.

TABLE II: Rate of the β-Alanine Return Reaction at Various pH Values and in Various Buffers.

pH	rate of the reverse reaction[a] (μmol min^{-1} (mL of reaction mixture)$^{-1}$) phosphate	Tes	Tris
7.2	0.135	0.240	0.290
7.5	0.240	0.255	0.290
7.8	0.315	0.305	0.305
8.1	0.350	0.350	0.330
8.4	0.390		0.330

[a] The reaction mixture contained 20 mM β-alanine. The buffers were 3 mM Tris (H_2SO_4), 5 mM Tes (KOH), and 20 mM potassium phosphate. The rates were measured from the slopes of the curves like Figure 6b at "2 μmol/mL of β-alanine moved to pantothenate".

Figure 7a presents further results showing the dependence of the nonlinearity of the progress curve on the pK value of the buffer, and Figure 7b shows the corresponding curves for the reverse reaction. At pH 8, with low pK values the curvature is strong and the reverse reaction is rapid, and the order of the curves follows the order of the pK values. Measurements at higher pH values show that the pK effect is not so prominent above pH 8 as between pH 7 and 8.

Effect of Buffer Concentration. Figure 8 shows the effect of Tris buffer concentration on the forward and reverse reactions. At high buffer concentrations the forward reaction becomes more rapid and the β-alanine return reaction becomes slower. Similar buffer concentration effects can be detected also with other buffers, e.g., phosphate.

Both the pK and buffer concentration effects suggest that the real component of the buffer affecting the rate is the form with bound H^+ ion (HA but not A^-). At a constant pH the concentration of HA is changed with the buffer concentration. At various pH values the HA concentration is essentially changed only at the basic side of the pK value, where also the pH effect on the progress curve is detectable. The effect of HA means a general acid catalysis in some stage of the enzymatic reaction.

Although the acidic form of the buffer seems to be the main component affecting the rate, also the basic form has some effects. If the HA concentration remains constant but the A^- concentration (or pH) increases, an increase is detected in the return reaction of β-alanine. For instance in phosphate buffer, when HA concentration was adjusted to 3 mM, the velocities of the return reaction were measured as 33, 48, 100, and 140 nmol/(min mL) at pHs 7.4, 7.7, 8.0, and 8.3, respectively. Similar effects are detected in Tris and triethanolamine buffers, too. The pH itself has no real effect on the rate of the return reaction (Figure 6b), so the above effect must be ascribed to A^- concentration. A^- cannot, however, be the main component affecting the rate in experiments of Figures 4–8 because then, for instance, the results in Figure 7 would be contradictory to Figure 8.

The existence of the general acid catalysis is further supported by Figure 9 in which the effects of the added bases depend on their pK values. The more the pK value of the added compound deviates from the measurement pH, the less effective the compound is as an acid catalyst.

Delay of the Reverse Reaction. The rate of the reverse reaction is at the beginning only about 1/8 of the final rate, but increases within a few minutes to the normal (Figure 6b). The delay is found near pH 7; above pH 8 it is insignificant. Also the forward reaction is somewhat slower at the beginning of the progress curve (Figure 6a). The delay is increased by dilution of the enzyme. In 2 mM phosphate buffer (pH 7) the delay lasts about 3, 6, and 14 min at relative enzyme concentrations 1, 0.5, and 0.25, respectively. An increase in phosphate

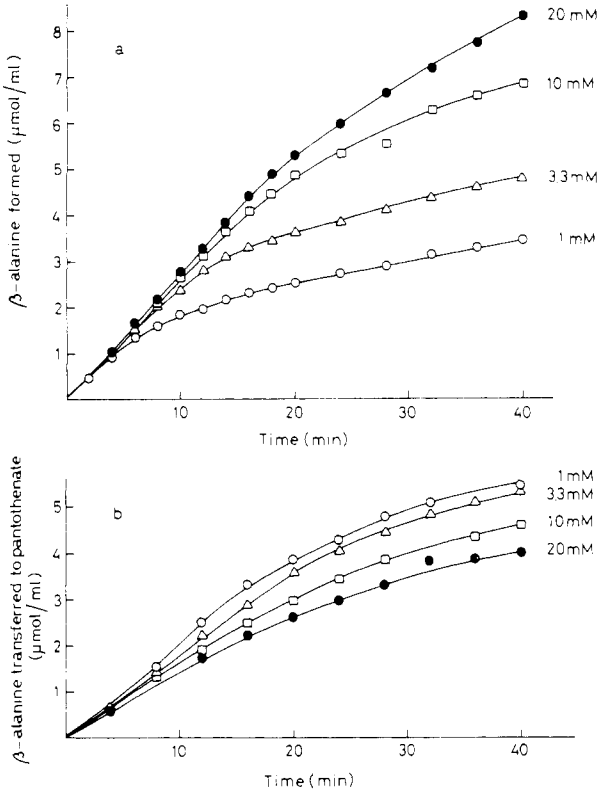


FIGURE 8: Effect of buffer concentration on the progress of pantothenate hydrolysis (a) and on the return reaction of β-alanine (b). The buffer was Tris (H_2SO_4), pH 8, the concentrations as indicated.

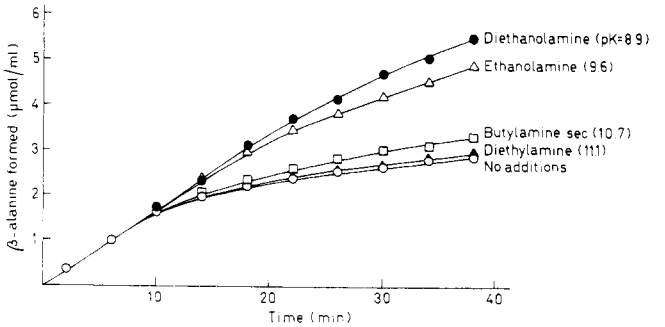


FIGURE 9: Effect of various bases with high pK values on the progress of pantothenate hydrolysis by pantothenase. The compounds were at 20 mM concentration, and their pH was adjusted to 8.0 with H_2SO_4. The pH of the reaction mixtures was fixed with 2 mM Tricine (H_2SO_4), pH 8.0 (pK = 8.15).

concentration causes a remarkable increase in the delay; at 50 mM phosphate (pH 7) no real change can be detected in the rate of the reverse reaction.

The reason for the delay remains obscure. The time dependence, in addition to the difference in the forward and reverse reactions, suggests some kind of structural change, either a conformational change or a subunit structure change. Direct measurements of the tryptophan fluorescence (excitation wavelength at 280 nm and emission wavelength at 332 nm) did not, however, provide any distinct evidence of a conformational change. In 3 mM Mops buffer (pH 7.2) the tryptophan fluorescence emission remained unchanged for 6 min and thereafter began to fall at the rate of 0.9%/min. At 6 min the reverse reaction has reached its normal velocity. At pH 8 a slight fall of the tryptophan fluorescence emission is detectable from the beginning.

The delay of the reverse reaction disturbs the kinetic measurements of the forward and reverse reactions. Above pH 8 the progress curve follows the parabolic shape derived from eq 3. Between pH 7 and 8 the delay of the reverse reaction causes a linear part (or curved upward) to the beginning of the progress curve and only thereafter the parabolic rule is followed. The parabolic part is then shifted upwards more the longer the delay stays (Figure 4).

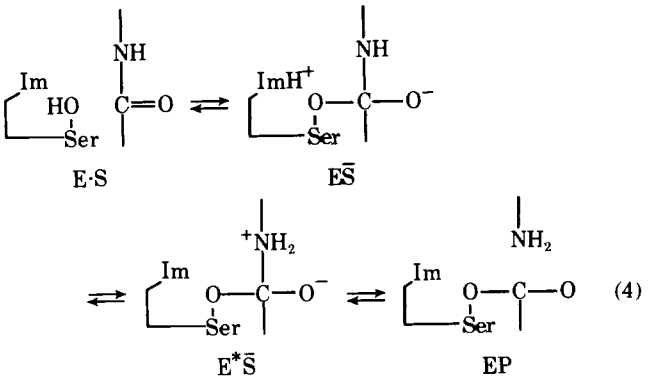
Inhibition with m-Aminophenylboronic Acid. Various boronic acids are inhibitors of the serine proteases, and some information on the mechanism of enzymic catalysis has been derived by using these inhibitors (Philipp & Bender, 1971; Koehler & Lienhard, 1971; Lindquist & Terry, 1974). The inhibition of pantothenase with *m*-aminophenylboronic acid was tested as one piece of evidence on the similarity of pantothenase to the serine proteases. Pantothenase was competitively inhibited with the boronic acid; the K_i value at pH 7 was 0.1 mM. With chymotrypsin and subtilisin the K_i values of the boronic acid inhibitors are of the same order of magnitude (Philipp & Bender, 1971).

Discussion

Many kinetic similarities exist between the pantothenase reaction and the reactions of the serine proteases. The bond to be broken is an amide bond. The pH dependence of the pantothenase reaction is similar to that of the serine proteases (Bender & Kezdy, 1965; Goldstein, 1972). Both the pK and ΔH of the ionizable group affecting the pantothenase reaction suggest that it is a histidine imidazole (Airas, 1976); an imidazole is known to be present at the active site of the serine proteases (Bender & Kezdy, 1965). Pantothenase is inactivated by phenylmethanesulfonyl fluoride, a reagent that is bound to serine OH groups in proteins (Fahrney & Gold, 1963). Also the above inhibition with 3-aminophenylboronic acid is a similarity between pantothenase and the serine proteases. The boronic acid bound to the enzyme is said to form an analogue of the tetrahedral intermediate of the serine protease reaction (Philipp & Bender, 1971; Koehler & Lienhard, 1971; Lindquist & Terry, 1974). An acyl-enzyme has been shown to be an intermediate during the reaction of the serine proteases (Bender & Kezdy, 1965; Kraut, 1977), and the existence of the return reaction of β-alanine suggests an acyl-enzyme intermediate for pantothenase. Although the exact structure of the active site of pantothenase remains still unexamined, the many common features of the kinetics suggest also similar reaction mechanisms for pantothenase and the serine proteases. Some direct evidence is available on the involvement of an OH group in addition to the imidazole group in the reaction and also on the formation of an acyl-enzyme, but the existence of a tetrahedral intermediate during the pantothenase reaction is mainly an assumption based on the similarities.

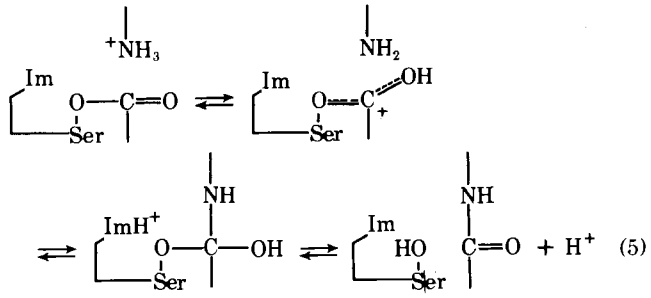
Normally the serine protease reaction with amide substrates has not been found to be reversible even from the stage of acyl-enzyme (Kraut, 1977). With ester substrates such a reversibility exists. However, also with amide substrates some reversibility may be found from the stage of acyl-enzyme, if the leaving group is an amino acid amide or a peptide (Fastrez & Fersht, 1973). β-Alanine is not an amide in the return reaction, and this is thus a difference between the pantothenase and serine protease reactions. The acyl-enzyme formation is the rate-limiting step of the serine protease reactions with amide substrates (Bender & Kezdy, 1965; Kraut, 1977), and also with pantothenase the rate limitation occurs at the acylation.

The mechanism of acylation of α-chymotrypsin has been suggested to be the following (Satterthwait & Jencks, 1974):



(4)

The reverse reaction begins with a nucleophilic attack of $-NH_2$ to the acyl carbon atom. However, when applied to pantothenase, the concentration of the $-NH_2$ form of β-alanine is dependent on pH between pH 7 and 8.5 (pK = 10.2). Thus the rate of the reverse reaction should be dependent on pH. The independence of pH would suggest that the ionic form $-NH_3^+$, is the reacting one. The detailed mechanism of the reverse, aminolytic reaction could be written as eq 5:



(5)

The aminolytic reaction begins with a proton transfer to the acyl group (an acid catalytic step) and then a nucleophilic attack follows. The main difference between eq 4 and 5 is that at the stage of the tetrahedral intermediate the imidazole is uncharged in the former but charged in the latter. This difference could be thought to determine the bond to be broken.

The role of the general acid catalysis caused by the buffers remains still quite obscure. Neither the exact mechanism nor even the intermediate step of the reaction including the acid catalysis is known. The acid catalysis both stimulates the forward reaction and decelerates the return reaction of β-alanine. The acid catalysis therefore cannot play a role in the formation of the acyl-enzyme because the resulting increase in the acyl-enzyme concentration would accelerate both of the reactions mentioned. Any acid catalysis causing an inhibiting effect on the return reaction of β-alanine is hard to imagine. An acid catalysis in the hydrolysis of the acyl-enzyme seems to be a possible explanation because an increase in the deacylation causes a decrease in the acyl-enzyme concentration and thus a decrease in the rate of the β-alanine return reaction. As the rate-limiting step is at the acylation, the acid catalysis in the deacylation could not affect the initial rate of the forward reaction. Thus the acid catalysis could not be seen before a sufficient concentration of β-alanine is formed. This is consistent with the results in Figures 7a and 8a.

If it is assumed that the acid catalysis occurs in the deacylation, there still exist various possibilities for the reaction mechanism. The acid catalysis can occur either before or after the nucleophilic attack of water to the acyl carbon. The former case would represent a general scheme of the bimolecular, acid-catalyzed ester hydrolysis (Bender, 1960), and the resulting detailed mechanism of such an acyl-enzyme hydrolysis is given in eq 6.

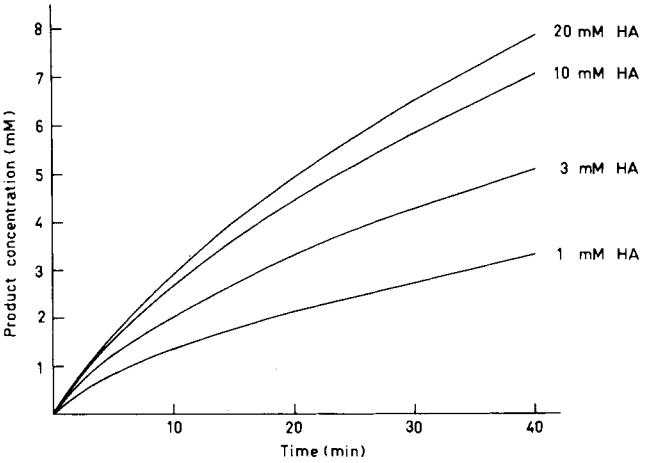
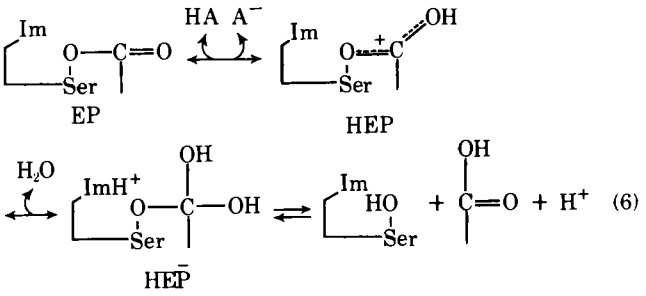


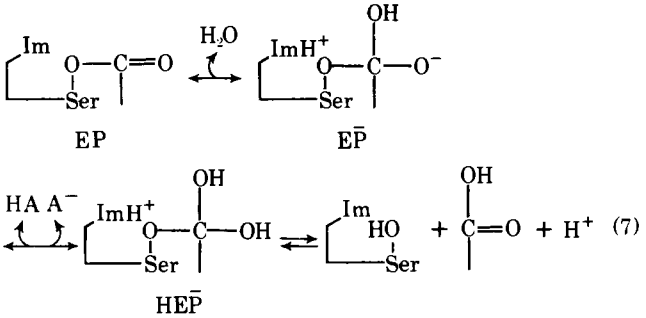
FIGURE 10: Computed curves of the effect of the "acid" concentration on the reaction velocity curves. The calculation was based on the integrated form of eq 9 (corresponding to eq 3). The reaction velocity constants used in the calculations were as follows: $k_{+4} = k_{+7} = k_{+9} = 1 \times 10^9$ (s^{-1}), $k_{+2} = 15$ (s^{-1}), $k_{-2} = 1 \times 10^4$ (s^{-1}), $k_{+3} = 1 \times 10^4$ (s^{-1}), $k_{+5} = 1 \times 10^4$ (s^{-1} M^{-1}), $k_5 = 1 \times 10^6$ (s^{-1} M^{-1}), $k_{+6} = 1 \times 10^4$ (s^{-1}), $k_{+8} = 2 \times 10^4$ (s^{-1} M^{-1}). The enzyme concentration value used was $e = 1 \times 10^{-6}$ M. The "acid" concentrations were 1, 3, 10, and 20 mM, as indicated, and the A^- concentrations were equal to the HA concentrations.



(6)

The deacylation begins with proton transfer to the acyl group. The reaction is assumed to be reversible, and thus A^- affects also the acyl-enzyme concentration. The nucleophilic attack of water to the modified acyl group follows the acid catalytic step. Equation 6 is analogous to the aminolytic reaction of eq 5.

A kinetically indistinguishable mechanism from eq 6 is derived if the nucleophilic attack is assumed to occur before the acid catalysis (eq 7).

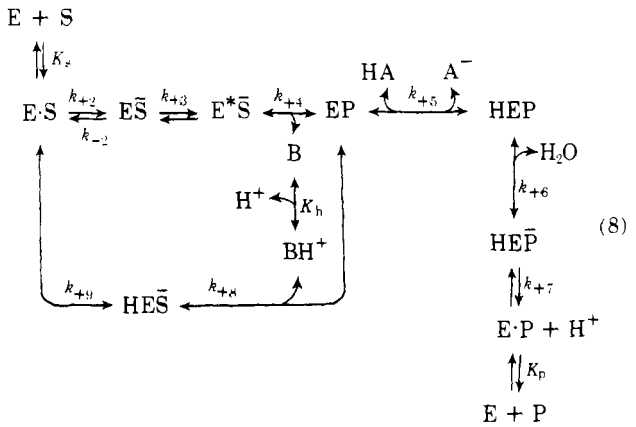


(7)

The mechanism of eq 6 may, however, be chosen in preference to eq 7 because the degradation of HEP̄ is apparently a fast reaction, and in eq 7 the effect of A^- on the acyl-enzyme (or on the return reaction of β-alanine) would then become impossible.

The resulting total mechanism of the pantothenase catalyzed hydrolysis of pantothenic acid is described in eq 8. This contains the mechanisms of eq 4–6; thus the acid catalysis occurs

in the deacylation before the nucleophilic attack of water, and β-alanine reacts in its return reaction in the $-NH_3^+$ form.



(8)

The reaction mechanism of eq 8 gives rise to the steady state reaction velocity eq 9. The substrate binding reaction, the product dissociation reaction, as well as reaction $H^+ + B \rightleftharpoons BH^+$ are assumed to be in equilibria, and their reaction velocity constants are omitted. The reverse reaction velocity constants of reactions 3, 4, 6, 7, 8, and 9 are omitted as well, because the forward reactions 4, 7, and 9 are assumed to be very fast, and the above reverse reactions would then become insignificant.

$$v = \frac{e}{c_1 B + c_2} \tag{9}$$

where e = total enzyme concentration

$$c_1 = \frac{k_{+8}}{k_{+5}[HA]}\left(1 + \frac{k_{-5}[A]}{k_{+6}}\right)\left(\frac{1}{k_{+2}} + \frac{1}{k_{+3}} + \frac{1}{k_{+4}} + \frac{1}{k_{+9}} + \frac{k_{-2}}{k_{+2}k_{+3}}\right)$$

$$c_2 = \frac{1}{k_{+2}} + \frac{1}{k_{+3}} + \frac{1}{k_{+4}} + \frac{1}{k_{+6}} + \frac{1}{k_{+7}} + \frac{k_{-2}}{k_{+2}k_{+3}} + \frac{1}{k_{+5}[HA]} + \frac{k_{-5}[A]}{k_{+6}k_{+5}[HA]}$$

Equation 9 is of the same form as eq 2 and thus the progress curve is parabolic.

Figure 10 shows computed curves derived from the mechanism of eq 8. The curves are quite similar to the experimental curves in Figure 8a. Although the real reaction velocity constants are not known, Figure 10 shows that it is possible to estimate the reaction velocity constants so that eq 9 explains the experimental results.

References

Airas, R. K. (1976) *Biochem. J. 157,* 415.

Airas, R. K., Hietanen, E. A., & Nurmikko, V. T. (1976) *Biochem. J. 157,* 409.

Bender, M. L. (1960) *Chem. Rev. 60,* 53.

Bender, M. L., & Kezdy, F. J. (1965) *Annu. Rev. Biochem. 34,* 49.

Fahrney, D. E., & Gold, A. M. (1963) *J. Am. Chem. Soc. 85,* 997.

Fastrez, J., & Fersht, A. R. (1973) *Biochemistry 12,* 2025.

Goldstein, L. (1972) *Biochemistry 11,* 4072.

Koehler, K. A., & Lienhard, G. E. (1971) *Biochemistry 10,* 2477.

Kraut, J. (1977) *Annu. Rev. Biochem. 46,* 331.

Lindquist, R. N., & Terry, C. (1974) *Arch. Biochem. Biophys. 160,* 135.

Philipp, M., & Bender, M. L. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68,* 478.

Satterthwait, A. C., & Jencks, W. P. (1974) *J. Am. Chem. Soc. 96,* 7018.

Binding of Bovine Coagulation Factor X_a to Platelets†

Björn Dahlbäck and Johan Stenflo*

ABSTRACT: The binding of highly purified bovine coagulation factor X_a to washed bovine platelets was studied. ^{125}I-labeled factor X_a underwent binding to a platelet receptor that became accessible only after induction of the platelet release reaction by thrombin or by the calcium ionophore A 23187. The zymogen factor X did not bind to platelets. The factor X_a binding was saturable, reversible, and correlated with the rate of thrombin formation. The number of factor X_a binding sites per platelet was 290–420 and the apparent association constant was estimated to be 2.8×10^9 to 1.0×10^{10} M^{-1}. Diisopropyl fluorophosphate–factor X_a bound to the same platelet receptor as factor X_a indicating that limited proteolysis of a receptor protein was not required for binding. The rate of factor X_a binding was rapid (2.1×10^6 to 2.9×10^6 M^{-1} s^{-1}) and similar to that previously found for the rate of binding of polypeptide hormones to their receptors. Displacement of factor X_a from the platelet receptor by diisopropyl fluorophosphate–factor X_a effectively blocked thrombin formation. Low concentrations of factor X_a catalyze prothrombin activation more effectively in the presence of platelets than in the presence of phospholipid and factor V.

Platelets have a central role in hemostasis (Weiss, 1975; Gordon, 1976). Subsequent to vascular injury, platelets adhere to subendothelial collagen and aggregate to each other forming a so called hemostatic plug. This process is accompanied by a release reaction, i.e., exocytosis of platelet granules with release of both high and low molecular weight components such as fibrinogen, Ca^{2+}, serotonin, and adenosine disphosphate. The aggregated platelets appear to provide a catalytic surface for localized activation of the plasma clotting factors. This

† From the Department of Clinical Chemistry, University of Lund, Malmö General Hospital, S-214 01 Malmö, Sweden. *Received April 25, 1978.* This investigation was supported by the Swedish Medical Research Council (Project No. B78-13X-04487-04B) and Direktör Albert Påhlssons Stiftelse.

0006-2960/78/0417-4938$01.00/0 © 1978 American Chemical Society