Purification of Biotinidase from Human Plasma and Its Activity on Biotinyl Peptides†

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ABSTRACT: Biotinidase catalyzes the hydrolysis of N^{ϵ}-biotinyllysine (biocytin) to form biotin and free lysine. The enzyme has been purified 4800-fold from outdated human plasma and was determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis to have a molecular weight of $(76 \pm 2) \times 10^3$. The same molecular weight was found by molecular sieve chromatography under nondenaturing conditions, indicating biotinidase is a monomer. This value is in contrast to a molecular weight of 115 000 determined by Pispa [Pispa, J. (1965) *Ann. Med. Exp. Biol. Fenn., Suppl. 5*, 5–39] with an impure biotinidase. The K_m for biocytin was 6.2×10^{-6} M, and biotinidase was found to be sensitive to phenylmethanesulfonamide and iodoacetamide in agreement with earlier studies by Knappe and co-workers [Knappe, J., Brümmer, W., & Bierderbick, K. (1963) *Biochem. Z. 338*, 599–613], who suggested that serine hydroxyl groups and sulfhydryl groups are essential for enzymatic activity. The specificity of biotinidase was examined by using synthetic and natural biotinyl peptides isolated by specific proteolytic cleavage of the biotinyl subunit of transcarboxylase. It was found that the rate of hydrolysis of biocytin was 83-fold higher than that found for biotin-containing peptides 5–13 residues in length. Removal of methionine from either side of the conserved region around the biocytin did not greatly alter the rate of cleavage. Increasing the peptide to 65–123 residues in length decreased the rate 1200-fold compared to that of biocytin. For these determinations, a procedure was developed for separating the released biotin from uncleaved biotinyl peptides and apopeptides (peptides with biotin removed) by using reverse-phase high-performance liquid chromatography. It is concluded that the primary substrate of biotinidase in vivo is biocytin or very short biotinyl peptides.

As part of a program to investigate the addition and release of biotin from biotin-containing enzymes, we have purified biotinidase (EC 3.5.1.12) from outdated human plasma. The reaction involved in the hydrolysis of biocytin or biotinyl peptides is illustrated in Figure 1. The hydrolysis is of interest for three reasons: (1) the specificity of biotinidase for different substrates, (2) the mechanism by which biotin is released from biotin enzymes and the subsequent turnover of biotin into newly synthesized biotin enzymes, and (3) the utilization of biotinidase to prepare apobiotin peptides for use as substrates in the study of the specificity of the biotin holoenzyme synthetase. This enzyme catalyzes the covalent attachment of biotin to the ϵ-amino group of a specific lysine residue of biotin enzymes.

Biotinidase was first described by Thoma & Peterson (1954). They found that biotinidase was only capable of releasing biotin from hog liver extracts which had been pretreated with trypsin. This observation indicated that biotinidase is not active when biotin is attached to large native proteins. Evidence supporting this postulate was offered by Koivusalo et al. (1963), who found that biotinidase from *Streptococcus faecalis* was unable to release biotin from intact propionyl-CoA carboxylase.

The mechanism of biotinidase with *N*-biotinyl-*p*-aminobenzoate as a substrate was investigated by Knappe et al. (1963), who observed that, in the presence of hydroxylamine, biotin hydroxymate was produced. These results suggested that the intermediate products of the hydrolysis are free lysine and acylbiotin derivative of the enzyme. The intermediate is probably in the form of an acylbiotin–sulfhydryl enzyme complex that is subsequently hydrolyzed in the presence of water to release the biotin. When radioactive [^{14}C]lysine was included in the assay, [^{14}C]biocytin was detected, which indicated that [^{14}C]lysine can exchange into the intermediate complex and that the reaction is reversible at the level of this high-energy intermediate. There is, however, no evidence to suggest that biotinidase can catalyze the overall reverse reaction, i.e., the formation of biocytin from lysine and free biotin.

Although the existence of biotinidase in mammalian tissues and microorganisms has been known for 30 years, it has never been purified to homogeneity, and its activity on biotinyl peptide per se has never been investigated. The present investigation addresses the question whether biotin enzymes must be completely degraded to biocytin before biotinidase acts or whether biotinidase can remove biotin from small biotinyl peptides. For this purpose, we have purified biotinidase and determined its activity on synthetic biotinyl peptides and on biotinyl peptides isolated from transcarboxylase of *Propionibacterium shermanii*.

Experimental Procedures

Materials

[^{14}C]Biotin was obtained from Amersham, [^{3}H]biotin from New England Nuclear, affinity-purified avidin from Sigma Chemical Co., malate dehydrogenase from Boehringer-Mannheim, and tosylphenylalanine chloromethyl ketone (TPCK)[1]-trypsin from Worthington Biochemical Corp.

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[1] Abbreviations: BCT, biocytin; HPLC, high-performance liquid chromatography; SDS, sodium dodecyl sulfate; TPCK, tosylphenylalanine chloromethyl ketone; EDTA, ethylenediaminetetraacetic acid.

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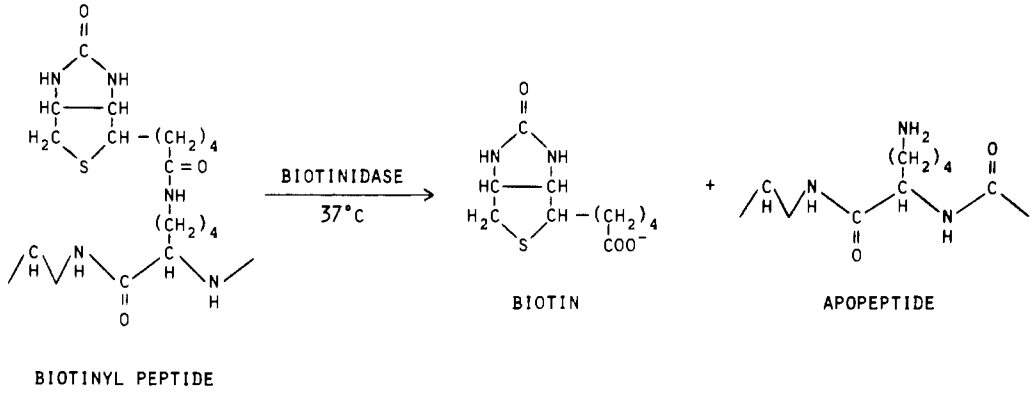


FIGURE 1: Action of biotinidase on biocytin or biotinyl peptides.

Proline-specific endopeptidase and *Staphylococcus aureus* V_8 protease were from Miles Biochemicals, CNBr-activated Sepharose 4B was from Pharmacia Fine Chemicals, micropolyamide plates were from Schleicher & Schuell, and a TSK-250 HPLC gel filtration column and silver stain kit were from Bio-Rad. The C_{18} reverse-phase column was from Synchrom, Inc., HPLC-grade trifluoroacetic acid from Pierce Chemical Co., and HPLC-grade ultrapure acetonitrile from MCB Reagents. All other chemicals used in this work were analytical grade or better.

Methods

Synthesis of N-Biotinyl-p-aminobenzoic Acid. The artificial substrate *N*-biotinyl-*p*-aminobenzoic acid was synthesized by the method of Knappe et al. (1963) using ethyl chloroformate. The protocol was scaled up 10-fold with routine yields of 70%. Rotary evaporation was performed at 55 °C. The substrate was dissolved in 5% sodium bicarbonate and stored at −20 °C.

Assay of Enzyme. Biotinidase activity was determined by the method of Knappe et al. (1963). The reaction mixture contained, in micromoles in a total volume of 1 mL, potassium phosphate (100) pH 6.0, EDTA (10), *N*-biotinyl-*p*-aminobenzoic acid (0.15), and 0.25 mg of serum albumin, and the enzyme solution to be determined. After a 30-min incubation at 37 °C, the reaction was stopped with 0.1 mL of 30% trichloroacetic acid and the protein removed by centrifugation. The supernate, 0.75 mL, was added to 0.25 mL of H_2O plus 0.1 mL of freshly prepared 0.1% sodium nitrite and incubated 3 min. Excess nitrite was then destroyed with 0.1 mL of 0.5% ammonium sulfamate followed by a 3-min incubation. *N*-(1-Naphthyl)ethylenediamine dihydrochloride (0.1 mL, 0.1%) was then added, and the absorbance at 546 nm was measured after 10 min. An assay mixture without enzyme was used as a control. A standard curve relating absorbance units to nanomoles of *p*-aminobenzoic acid was established and found to be linear to an optical density of 1.1. By definition, 1 milliunit of enzyme corresponds to the formation of 1 nmol of *p*-aminobenzoic acid (or biotin)/min from *N*-biotinyl-*p*-aminobenzoate. The assay was found to be linear up to 60 min with 1.5 milliunit of enzyme.

Protein Determination. Protein concentration was determined by using the method of Warburg & Christian (1941) as described by Layne (1957).

Peptide Detection and Determination. Peptide content was routinely monitored by measuring absorbance at 220 nm. Natural biotinyl peptides isolated from transcarboxylase of *P. shermanii* grown in the presence of [^{3}H]biotin were also assayed by radioactivity. When greater accuracy was required, the concentration of the peptides were determined by amino acid analysis.

N-Terminal Determination. N-Terminal determinations were carried out by using the dansylation procedure described by Bruton & Hartley (1970) except that the dansyl peptides were hydrolyzed for 7 h at 105 °C. The dansyl amino acid was identified by using the method of Hartley (1970) except that toluene–acetic acid (9:1 v/v) was used as the second developing solvent.

Amino Acid Analysis. Amino acid compositions were determined on either a Durrum D-500 or a Beckman 119 CL analyzer. Hydrolysis was performed with 200 μL of constant boiling 6 N HCl and 0.1% β-mercaptoethanol at 110 °C for 24 h in vacuum sealed tubes.

SDS Gel Electrophoresis. SDS–polyacrylamide gel electrophoresis was performed in 0.75-mm vertical slab gels, 7.5% in acrylamide as described by Laemmli (1970). When higher sensitivity was desired, gels were silver stained as described by Merril et al. (1979).

Preparation of Biotinyl Peptides. The biotinyl peptides were obtained by proteolytic cleavage of the biotinyl subunit of transcarboxylase which has been sequenced (Maloy et al., 1979). The complete $1.3S_E$ biotinyl subunit (123 residues) was isolated as described by Maloy et al. (1979). The 65-residue peptide was obtained by cleavage of the biotinyl subunit with a proline-specific endopeptidase by the method of Yoshimoto et al. (1980). Under the conditions used, the endopeptidase cleaves primarily Pro–Ala bonds. For the 24-residue peptide, the cleavage was with TPCK–trypsin according to the method of Maloy et al. (1979). The five-residue peptide was obtained by cleavage with *Staphylococcus aureus* V_8 protease at pH 7.8 according to the method of Austen & Smith (1976). The most efficient cleavage of Glu–Thr bonds is at this pH. The 13- and 12-residue peptides were chemically synthesized by the methods of Bodanszky et al. (1979, 1980). The large peptides 24 and 65 residues were initially fractionated by chromatography on Sephadex G-50 (1 × 100 cm) and the 5-residue peptide was fractionated on Sephadex G-25 (1 × 90 cm) each equilibrated with 5% acetic acid. After lyophilization, the eluates were loaded onto a Synchropak 300-Å pore size C_{18} column (250 × 4.1 mm) at a flow rate of 0.9 mL/min. Solvent A was 0.05% trifluoroacetic acid, and solvent B was acetonitrile containing 0.05% trifluoroacetic acid. Elution was first with 100% solvent A for 10 min followed by a gradient of 0–40% solvent B for 30 min. For the 65- and 123-residue peptides, the elution was with 100% solvent A for 10 min and a gradient of 0–80% solvent B for 36 min. The chemically synthesized peptides were purified by using HPLC but without prior Sephadex chromatography. To identify each peptide, the composition and N-terminal were determined and compared with the known sequences of the $1.3S_E$ subunit (Maloy et al., 1979).

Cleavage of Biotin from Biotinyl Peptides and Determination of Rate of Cleavage. The reaction mixture contained, in 200–400 μL of potassium phosphate (20 μmol), pH 6.0, and EDTA (2 μmol), 10–100 μg of biotinidase purified from DE-52 cellulose (~180 milliunits/mg) and 10 nmol of the appropriate biotinyl peptide. The concentration of biotinidase was varied depending on the rate of hydrolysis of the peptide. The reactions were stopped by heating the mixture at 100 °C for 5 min, and the resulting biotin, apopeptide, and residual biotinyl peptide were separated by using HPLC with solvents A and B, as described above. Two separate programs were usually used. The column was eluted for 10 min with solvent A followed by a linear gradient (i) 0–40% solvent B for 30 min or (ii) 3–32% solvent B for 24 min.

Determination of Biotin. The fractions where biotin eluted from the HPLC were quantitated by radiochemical assay using [^{14}C]biotin and avidin as described by Rylatt et al. (1977). Rylatt's assay was found to be linear over the range 10–100 pmol of biotin. From the amount of biotin in the fraction, the nonomoles of biotin cleaved per minute per milligram of biotinidase was estimated. Pure biotinidase was found to have a specific activity of 360 milliunits/mg of protein. If enzyme of lower specific activity was used for the cleavage, the value was adjusted. For example, if the specific activity of the biotinidase was 180, the observed milliunits per milligram were multiplied by 2.

Purification of Biotinidase. Outdated whole blood (4 L) which had been pretreated with sodium citrate was centrifuged at 4 °C in 250-mL aliquots for 10 min at 1500*g*. The pellets contained negligible amounts of biotinidase and were discarded. The supernatant fractions were combined, and all subsequent operations were performed at 4 °C.

Ammonium Sulfate Fractionation. Biotinidase precipitates between 35 and 58% saturation with ammonium sulfate. Solid ammonium sulfate was added to a concentration of 35% to the plasma from 4 L of blood, and the precipitated protein was removed by centrifugation at 20000*g* for 30 min. The biotinidase was then precipitated by the addition of solid ammonium sulfate to 58% saturation, and the precipitate was collected by centrifugation as described above. The 58% pellet was dissolved in 1400 mL of 30 mM potassium phosphate, pH 7.0, containing 1 mM EDTA and 0.7 mM β-mercaptoethanol and dialyzed overnight at 4 °C against three 12.0-L changes of the same buffer.

DEAE-Sephadex Chromatography. The dialyzed solution was applied to a DEAE-Sephadex A-50 columm (5 × 60 cm) which had been equilibrated with the above buffer. The column was washed with 1 L of the buffer followed by 1 L of 80 mM potassium acetate, pH 5.2, containing 40 mM sodium chloride, 1 mM EDTA, and 0.7 mM β-mercaptoethanol. A 2-L linear gradient of sodium chloride from 40 to 400 mM in the above buffer was then applied to the column, and the biotinidase eluted at 200 mM sodium chloride. The fractions containing the enzyme were pooled and brought to 60% saturation with ammonium sulfate. The resulting precipitate was dissolved in 100 mL of 30 mM potassium phosphate, pH 7.0, containing 50 mM potassium chloride, 1 mM EDTA, and 0.7 mM β-mercaptoethanol.

Chromatography on Bio-Gel A-0.5m. The solution from the previous step was split into two equal aliquots and each applied separately to a Bio-Gel A-0.5m column (2.5 × 90 cm) which had been equilibrated with the above buffer. The enzyme eluted in the last of three overlapping protein peaks (data not shown). The fractions containing the enzyme from both runs were pooled and precipitated with a 60% saturation of ammonium sulfate. The precipitate was dissolved and dialyzed overnight at 4 °C against 2 L of the column buffer.

Table I: Levels of Biotinidase

source	specific activity, crude[c] (milliunits/mg)
hog kidney	0.038
fresh human blood serum[a]	0.138
outdated human blood serum[b]	0.075

[a] Obtained by venapuncture. [b] >35 days old; obtained from the American Red Cross. [c] Milliunits is nanomoles of *N*-biotinyl-*p*-aminobenzoate hydrolyzed per minute.

Affi-Gel Blue Chromatography. The above dialyzed solution in a volume of 10 mL was applied to an Affi-Gel Blue column (2.5 × 50 cm) which had been equilibrated with the above buffer. The biotinidase was eluted with this buffer, and the majority of the contaminating serum albumin remained bound. The pooled enzyme fractions were precipitated with a 60% saturation of ammonium sulfate, and the precipitate was dissolved and dialyzed overnight at 4 °C against 1 L of 30 mM potassium phosphate, pH 7.0, containing 1 mM EDTA and 0.7 mM β-mercaptoethanol.

DEAE-cellulose Chromatography. The dialyzed solution from the Affi-Gel Blue column in a volume of 6 mL was applied to a DEAE-cellulose (Whatman DE-52) column (1.1 × 38 cm) which had been equilibrated with the above buffer. The column was washed with 200 mL of 80 mM potassium acetate, pH 5.2, containing 50 mM potassium chloride, 1 mM EDTA, and 0.7 mM β-mercaptoethanol and then with 200 mL of 50–200 mM gradient of potassium chloride. Biotinidase eluted at ~130 mM potassium chloride. The fractions containing enzyme were pooled in a final volume of 265 mL and stored at 4 °C for further use. The enzyme lost activity with time when stored as an ammonium sulfate precipitate or as a concentrated protein solution and after 9 months had lost two-thirds of its activity.

HPLC Gel Filtration. When pure biotinidase of very high specific activity was required, a final purification was performed by HPLC. The fractions containing biotinidase were pooled and stored at −20 °C.

Results

Source of Biotinidase. Three sources of biotinidase were investigated. The results are summarized in Table I. The level of biotinidase from outdated plasma was approximately double that of a crude extract from homogenized hog kidneys. The biotinidase level in fresh plasma obtained by venapuncture was found to be approximately double that of outdated plasma. Due to the relatively high levels of biotinidase and the availability of outdated whole blood, the biotinidase was purified from this source.

Summary of the Purification of Biotinidase. The results of a typical purification of the enzyme are shown in Table II. The final step of purification was with HPLC (Figure 2). Recoveries ranged between 24 and 44%, yielding an average of 10 mg of purified enzyme/4 L of whole blood. This represents a 4800-fold purification of the enzyme. The final specific activity varied between 360 and 400 milliunits/mg. We have no explanation for the increase in total units after fractionation on DEAE-Sephadex. It may be indicative of an activating effect of the acetate buffer or removal of an inhibitory protein.

Determination of Purity and Molecular Weight of Biotinidase. The homogeneity of biotinidase which had been purified by HPLC (Figure 2) was assessed by SDS–polyacrylamide gel electrophoresis. A single band was observed (Figure

Table II: Summary of the Purification of Biotinidase from Human Plasma

purification step	volume (mL)	protein (mg)	activity (milliunits/mg)[c]	% recovery	overall purification
human blood plasma[a]	2540	169418	0.075	100	
35–58% ammonium sulfate	1400	142800	0.038	100	1.2
DEAE-Sephadex A-50	100	6357	2.36	118	31.5
Bio-Gel A-0.5m	10	275	24.95	74	333
Affi-Gel Blue	6	63	132.0	66	1760
DEAE-cellulose	26	29	180.0	41	2400
HPLC-TSK gel filtration[b]	0.05	0.025	361.4	36	4819

[a] Purification initiated with ~4000 mL of whole blood, outdated >35 days; a generous gift from the American Red Cross. [b] 1/500 of the enzyme from the DEAE-cellulose step was injected onto the HPLC column. [c] Milliunits is nanomoles of *N*-biotinyl-*p*-aminobenzoate hydrolyzed per minute.

Table III: Activity of Biotinidase on Biotinyl Peptides and Biocytin[a]

no.	no. of residues	residues 87 88 89 90 91 92 93	biotinidase act. (milliunits/mg)
1	123	1–86-Ala-Met-Bct-Met-Glu-Thr-Glu-94–123	0.06
2	65	59–86-Ala-Met-Bct-Met-Glu-Thr-Glu-94–123	0.07
3	24	78–86-Ala-Met-Bct-Met-Glu-Thr-Glu-94–101	0.30
4	13	Bct-Met-Glu-Thr-Glu-94–101	0.93
5	12	Met-Bct-Xxx-Xxx-Thr-Glu-94–101	1.2
6	5	Ala-Met-Bct-Met-Glu	1.2
7		biocytin	83

[a] The numbering of peptides is in accordance with the sequence of the biotinyl subunit of transcarboxylase (Maloy et al., 1979). For determination of biotinidase activity, the assay mixture (see Methods) was incubated at 37 °C for various times, as much as 24 h for the peptides giving very low activity (no. 1 and 2). Then the apopeptide, biotin, and residual holopeptide were separated by using HPLC, and the rate of cleavage was determined as described in the legend of Figure 4 and under Results.

3, top) which was estimated to have a molecular weight of 76 000 ± 2000. A molecular weight of 78 000 ± 5000 was obtained when molecular sieve chromatography was utilized to assess the size of biotinidase (Figure 3, bottom). In addition, both two-dimensional gel electrophoresis (nondenaturing in the first dimension and SDS–polyacrylamide gel electrophoresis in the second) and isoelectric focusing (data not shown) gave only a single protein band, confirming the purity of the preparation. The isoelectric point of biotinidase was 4.6. From these results, it is concluded that biotinidase is a monomer with a molecular weight of 76 000.

Determination of the Rate of Release of Biotin from Biotinyl Peptides by Biotinidase. Each peptide was incubated with biotinidase as described under Methods. The remaining uncleaved biotinyl peptide, apopeptide, and biotin were separated by using HPLC. An example for the pentapeptide is shown in Figure 4. Either the amount of biotinyl peptide cleaved to apopepetide was determined by measuring the mass of the areas under the apopeptide and residual biotinyl peptide peaks from the HPLC scan and calculating from these values the amount cleaved as illustrated in the legend of Figure 4 or the cleavage was determined from the amount of biotin in the biotin-containing fractions determined as described under Methods. The rate of release was linear with time (within experimental error). For example, calculations from the values obtained with the pentapeptide were 1.34 milliunits/mg at 4 h (experiment of Figure 4), and 1.10 milliunits/mg at 1 h. In a third experiment in which the cleavage was determined at 30 min from the biotin in fractions of the HPLC, the value was 1.24 milliunits/mg (average = 1.2 milliunits/mg). With the biocytin, the rate as determined from the biotin in the fractions from the HPLC (in milliunits/mg) was 71 at 10 min, 103 at 30 min, 84 at 40 min, and 87 at 50 min (average = 83 milliunits/mg).

The rates of release of biotin from the various biotinyl peptides are summarized in Table III. The decreasing order of cleavage efficiency is biocytin > pentapeptide > 12-residue peptide, 13-residue peptide > 24-residue peptide. Very low hydrolysis efficiency was observed with the peptides 1 and 2 of Table III. There were no shorter peptides found from the longer biotinyl peptide even after long periods of incubation with the biotinidase. Clearly, biotinidase has no peptidase activity. Each apopeptide was recovered and stored for studies with biotin holoenzyme synthetase. Due to the extremely low hydrolysis rates for peptides 1 and 2, the presence of these apopeptides could not be detected.

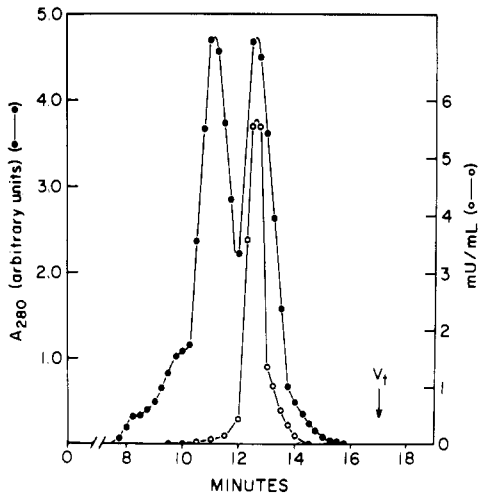
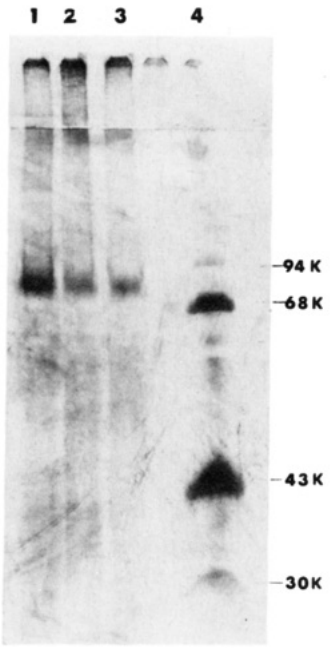


FIGURE 2: Purification of biotinidase on HPLC. Protein elution was monitored at A_{280} (●), and enzyme activity (○) was determined by assay with *N*-biotinyl-*p*-aminobenzoate. A 50-μL sample from the DEAE-cellulose column (see Table II) was chromatographed over Bio-Rad TSK-250 gel filtration column (7.5 × 300 mm) which had been equilibrated with 100 mM potassium phosphate, pH 7.0. At a flow rate of 0.6 mL/min and a pressure of 7 bar, the volume of the fractions was 0.25 mL.

Determination of the K_m for Biocytin and *N-Biotinyl-p-aminobenzoate.* The K_m for biocytin was estimated by incubating biotinidase with various micromolar concentrations of biotin (1, 2, 3, 4, 8, 16, and 25 at 37 °C) with 6 μg of biotinidase of specific activity = 180 (DEAE-cellulose of Table II) and by assaying at 1, 2, and 4 min the biotin content in fractions from the HPLC. The average values of biotin released (in nmol/min) were the following at the given concentrations of biocytin: 1 μM, 0.055; 2 μM, 0.091; 3 μM, 0.14;



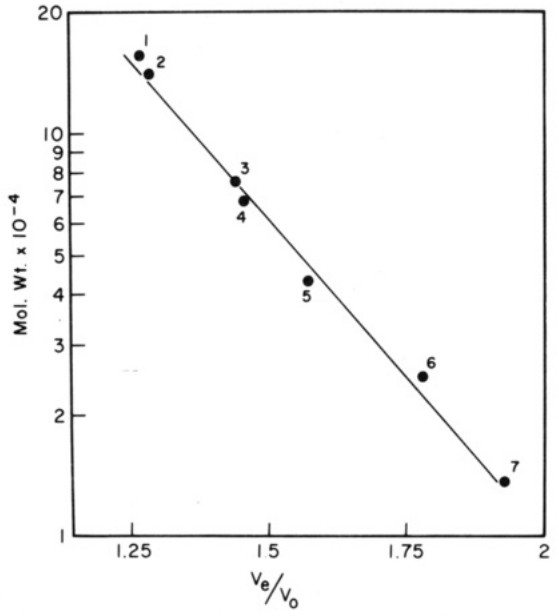


FIGURE 3: Determination of purity and molecular weight of biotinidase. (Top) Aliquots of the two peak fractions of biotinidase from HPLC of Figure 2 (lanes 1 and 2) and the following fraction (lane 3) were lyophilized and subjected to electrophoresis on a 7.5% polyacrylamide slab gel in the presence of 0.1% SDS. Lanes 1–3 contained 200, 125, and 80 ng of biotinidase, respectively. Lane 4 was 1 μg of Pharmacia low molecular weight standards. The standard proteins were phosphorylase B (M_r = 94 000), bovine serum albumin (M_r = 68 000), ovalbumin (M_r = 43 000), and carbonic anhydrase (M_r = 30 000). Silver staining was as described under Methods; it does not stain equal amounts of the standard proteins with equal intensity. (Bottom) Native molecular weight of biotinidase. All samples were run on a Sephacryl S-200 column (1.55 × 86 cm) at 4 °C in 30 mM potassium phosphate, pH 7.0, containing 1 mM EDTA and 0.7 mM β-mercaptoethanol. The location of biotinidase was determined by assay with *N*-biotinyl-*p*-aminobenzoate. The standard proteins used were (1) aldolase (M_r = 158 000), (2) lactate dehydrogenase (M_r = 140 000), (3) biotinidase, (4) bovine serum albumin (M_r = 68 000), (5) ovalbumin (M_r = 43 000), (6) chymotrypsinogen (M_r = 25 000), and (7) ribonuclease (M_r = 13 700). The molecular weight of biotinidase was determined to be 78 000 ± 5000.

4 μM, 0.17; 6 μM, 0.20; 8 μM, 0.21; 25 μM, 0.26. The $1/S$ vs. $1/V$ plot gave a K_m for biocytin of 6.25 μM and a V_{max} of 66.6 which corrected to a specific activity of 360 gives 133 milliunits/mg of biotinidase.

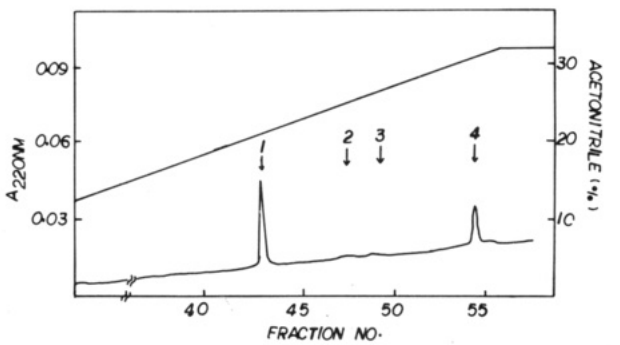


FIGURE 4: Separation of holopentapeptide from apopentapeptide and biotin using HPLC and determination of the rate of cleavage of the pentapeptide. The pentapeptide is incubated at 37 °C for 4 h with 50 μg of biotinidase purified on DEAE-cellulose (Table II) in a total volume of 200 μL as described under Methods. Apopentapeptide was in fraction 1, biotin in fractions 2 and 3, and residual holopentapeptide in fraction 4. Peaks 1 and 4 were scanned, and the apopeptide was found to be 67% of the sum of the two peaks. Accordingly, 6.7 nmol of the 10 nmol was cleaved in 4 h or 0.028 nmol/min by the 50 μg of biotinidase, i.e., 0.56 nmol min^{-1} mg^{-1}. The biotinidase had a specific activity of 150 milliunits, and on the basis of pure biotinidase (specific activity = 360), the milliunits per milligram = 1.34 [0.56(360/150)].

Similar studies were done with *N*-biotinyl-*p*-aminobenzoic acid using the spectrometric assay (see Methods). The K_m for *N*-biotinyl-*p*-aminobenzoic acid was 1.0×10^{-5} M. Biotin was found to be a competitive inhibitor of *N*-biotinyl-*p*-aminobenzoic acid with a K_i of 2.25×10^{-4} M.

Inhibition of Biotinidase. Biotinidase (2 milliunits; specific activity 180 milliunits/mg) was incubated with the inhibitor in 0.1 mL containing 3.0 mM potassium phosphate, pH 7.0, and 1 mM EDTA. Incubation was for 5 min with *p*-(hydroxymercuri)benzoate, 15 min with iodoacetamide, and 20 min with phenylmethanesulfonyl fluoride, and then the enzyme was assayed by using *N*-biotonyl-*p*-aminobenzoic acid as the substrate. The inhibition with *p*-(hydroxymercuri)benzoate was 19% at 5×10^{-8} M, 58% at 5×10^{-7} M, and 99% at 1×10^{-5} M. With iodoacetamide, there was 99% inhibition at 5×10^{-4} M and with phenylmethanesulfonamide, 99% at 1×10^{-7} M.

DISCUSSION

Biotin is a cofactor of key enzymes of carbohydrate and fatty acid metabolism and has an essential role. Since biotin is recycled in normal individuals the daily nutritional requirement is very low. Biotin occurs in foodstuffs and in the enzymes of the body in an amide linkage to the ε-amino group of lysine, and biotinidase is the only enzyme known that cleaves this bond. Recently, Wolf and co-workers (1983, 1984) have presented evidence that biotinidase deficiency is directly related to the late-onset form of multiple carboxylase deficiency. Patients diagnosed with the disease have extremely low levels of biotinidase activity in their serum and in some cases none at all. It has been found that these patients respond well to pharmacological doses of free biotin.

In spite of its importance, there is only limited information concerning biotinidase. The enzyme has never been purified to homogeneity, its activity on biotinyl peptides has not been investigated, and the possibility of a role in transport of biocytin or biotin in addition to its role in the hydrolysis of biocytin remains to be investigated.

Our studies have been directed toward purification of biotinidase and determination of its specificity for biocytin as compared to biotinyl peptides. It is generally considered that biotinidase can only act on biocytin (Utter & Sheu, 1980) and that biotin-containing proteins must be digested by proteases

and peptidases to biocytin before the biotin can be recycled by the action of biotinidase. The recycling also involves posttranslational attachment of the biotin to newly synthesized apoenzymes or subunits by holoenzyme synthetase.

It has been found that the sequence immediately surrounding the biocytin (biotinyllysin) is identical in all biotin enzymes so far sequenced including pyruvate carboxylase from sheep, turkey, and chickens (Rylatt et al., 1977), acetyl-CoA carboxylase from *Escherichia coli* (Sutton et al., 1977), and transcarboxylase from *P. shermanii* (Maloy et al., 1979). In all cases, an Ala-Met-Bct-Met sequence occurs, and in the pyruvate carboxylases and transcarboxylase the identity extends to Ala-Met-Bct-Met-Glu-Thr. There are several plausible reasons for this strict conservation: (1) the conserved region may serve as a recognition site for biotin holoenzyme synthetase to attach biotin to the appropriate lysine, (2) the region could serve to direct and facilitate the carboxylation and transcarboxylation reactions catalyzed by the biotin enzymes, or both, and (3) the region may play a role in the activity of biotinidase on biotinyl peptides.

To obtain homogeneous biotinidase, it was necessary to include an HPLC gel filtration to remove a bluish protein, thought to be ceruloplasmin, a copper-containing glycoprotein with an M_r 130000. Biotinidase has been reported to be a glycoprotein (Heard et al., 1985), and probable interaction of these two glycoproteins might be the reason for the difficulties in separating the M_r 76000 biotinidase from the M_r 130000 contaminant. The molecular weight of 76000 for biotinidase is consistent with the findings by B. Wolf, Virginia Commonwealth University, and co-workers (personal communication). Using serum from fresh blood, they have determined that biotinidase has a molecular weight of about 76000; these results indicate that the molecular weight of the biotinidase was not altered during the storage of the outdated blood which was used in our investigation.

The K_m value for biocytin which we determined to be 6.5×10^{-6} M is in agreement with another reported value of 5.0×10^{-6} M for biocytin of human serum (Koivusalo & Pispa, 1963). Pispa (1965) observed a K_m of 0.8×10^{-5} M with biotinidase from hog serum, and Knappe et al. (1963) observed a K_m of 1.6×10^{-5} M for the enzyme from hog kidney.

We find the enzyme is sensitive to sulfhydryl blocking reagents and also the serine group inhibitor phenylmethanesulfonamide. These results are consistent with previous studies by Knappe et al. (1963) and Koivusalo & Pispa (1963), who indicated these groups may play a role in the catalytic activity of biotinidase.

The results obtained on the relationship of the sequence of the biotinyl peptides to biotinidase activity lead to the following general observations: (1) with the longest peptides (peptides 1 and 2), the activity of biotinidase was very low; (2) with the shorter peptides, the removal of Met from either side of the biocytin on the biotinidase (peptide 6 compared to peptides 4 and 5 in Table III) did not affect the activity, which indicates the presence of these groups in the conserved region of the biotinyl subunit does not play a significant role in protecting the biocytin from the action of biotinidase; (3) the activity of the enzyme on biocytin far exceeded that of any of the peptides tested. We, therefore, conclude that the primary substrate of biotinidase in vivo is biocytin or perhaps very short biotinyl peptides. It is unlikely in view of the rates determined here that biotinidase acts on longer biotinyl peptides under physiological conditions.

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