Physicochemical Studies of Carboxypeptidase A Derivatives. II. *N*-Succinylcarboxypeptidase*

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ABSTRACT: Previous studies have shown that modification of active center tyrosyl residues of carboxypeptidase A with acetylimidazole markedly alters catalytic activity without discernible change in protein structure. Acetylation of *both* tyrosyl and lysyl residues with acetic anhydride induces similar catalytic changes but concomitantly alters protein structure (Bethune, J. L., Ulmer, D. D., and Vallee, B. L., *Biochemistry 3*, 1764 (1964)). The consequences of modifying amino groups only now have been assessed in an *N*-succinyl derivative of the enzyme. The structural stability of *N*-succinylcarboxypeptidase enzyme as a function of pH does not differ from that of the native enzyme as judged by optical rotatory dispersion, free-boundary electrophoresis, and enzymic activity. Sedimentation and viscometric studies, performed at pH 7.5, reveal no gross changes in shape, and no changes in protein conformation could be detected. *N*-Succinylcarboxypeptidase differs from the native enzyme only in charge-dependent properties, *i.e.*, electrophoretic mobility and dependence of $s_{20,w}$ on concentration.

Analysis of electrophoretic patterns suggests that succinylation of free amino groups occurs as a stepwise rather than a random reaction.

Bovine pancreatic carboxypeptidase A has proven a useful system for assessing relationships of protein structure and composition to enzymatic function. The enzyme exhibits remarkable alterations in catalytic rates, specificity, or both when subjected to a wide range of inorganic substitutions (Coleman and Vallee, 1960), and organic modifications such as acetylation (Simpson *et al.*, 1963; Riordan and Vallee, 1963), acylation with dicarboxylic acid anhydrides (Riordan and Vallee, 1964), iodination (Simpson and Vallee, 1966), coupling with 5-diazo-1H-tetrazole (Sokolovsky and Vallee, 1966, 1967), nitration with tetranitromethane (Riordan *et al.*, 1966), or irradiation with ultraviolet light (Piras and Vallee, 1966). In many instances, altered function has appeared to derive solely from modification of specific side chains of the active center[1] or substitution of specific metal ions at the active site of the enzyme. However, some reactions have been found to induce changes in protein secondary or tertiary structure and, in these, catalysis may be affected indirectly (Bethune *et al.*, 1964; Piras and Vallee, 1966).

Acetylation of tyrosyl residues of carboxypeptidase with acetylimidazole, for example, induces marked alterations in enzymatic activity but no discernible change in the physicochemical properties of the protein. In contrast, modification of both tyrosyl residues and amino groups with acetic anhydride causes both functional and structural pH-dependent instability (Bethune *et al.*, 1964).

N-Succinylcarboxypeptidase[2] hydrolyzes both peptide and ester substrates somewhat more rapidly than does the native enzyme but does not exhibit the changes in specificity characteristic of the acetylcarboxypeptidases prepared with acetic anhydride or acetylimidazole, Ac_A- or Ac_I-carboxypeptidase,[2] respectively. This derivative is prepared in a fashion identical with Ac_A-carboxypeptidase, resulting first in *N,O*-succinylcarboxypeptidase. However, modified carboxypeptidases arising from reaction with dicarboxylic acid anhydrides are unique in that *O*-acyltyrosine residues deacylate spontaneously, ultimately yielding enzymes in which only the amino groups are modified (Riordan and Vallee, 1964).

N-Succinylcarboxypeptidase was therefore chosen as a model system with which to assess the physicochemical consequences of modification of the free amino groups of the enzyme. The present studies indicate that the only

* From the Biophysics Research Laboratory, Department of Biological Chemistry, Harvard Medical School, and the Division of Medical Biology, Peter Bent Brigham Hospital, Boston, Massachusetts. *Received March 24, 1967.* This work was supported by Grant-in-Aid HE-07297 from the National Institutes of Health of the Department of Health, Education, and Welfare, U. S. Public Health Service.

† Research career development awardees of the National Institutes of Health, Department of Health, Education, and Welfare, U. S. Public Health Service.

[1] The designation "active site" will refer specifically to the nitrogen–metal–sulfur bond essential for hydrolysis. "Active center" will refer to all those features of primary, secondary, and tertiary structure, *including* the active site, which are required for substrate binding, specificity, or hydrolysis of substrate.

[2] Abbreviations used: Ac_A and Ac_I, acetylcarboxypeptidases prepared with acetic anhydride and acetylimidazole, respectively; *N,O*-succinylcarboxypeptidase denotes the derivative formed immediately after succinylation and *N*-succinylcarboxypeptidase that obtained after spontaneous desuccinylation of the *O*-succinyltyrosyl residues has been completed.

pertinent physicochemical differences between the succinyl and native protein are found in those properties which depend upon charge; further, they suggest a differential reactivity of the amino groups of the enzyme.

Materials and Methods

Five-times-recrystallized beef pancreas carboxypeptidase A_γ (Worthington Biochemical Corp., Freehold, N. J.), prepared by the method of Anson (1937), was washed three times with metal-free distilled water and dissolved in 2 M NaCl–0.02 M Veronal (pH 7.5) prior to use. It was then adjusted by dialysis to the ionic strength and pH desired. All chemicals employed were of reagent grade and used without further purification.

Peptidase activity was determined using the synthetic substrate carbobenzoxyglycyl-L-phenylalanine (Mann Research Laboratories) as described previously (Coleman and Vallee, 1960), and is expressed as an apparent proteolytic coefficient, C, defined as $\log a_0/a$ per minute per micromole of enzyme, where a_0 and a represent the concentration of substrate at time zero and time t, respectively (Simpson *et al.*, 1963). The assays were carried out at 0° in 1.0 M NaCl–0.02 M Veronal (pH 7.5); C was calculated from the linear portion of the first-order plots, observed when hydrolysis did not exceed 15%.

Esterase activity was determined as previously described (Simpson *et al.*, 1963). Assays were performed at 25° with 5 ml of 0.01 M hippuryl *dl*-β-phenyllactate in 0.2 M NaCl–0.005 Tris-HCl (pH 7.5). Activities are expressed as zero-order velocity constants, k, with units of moles of H^+ released per minute per mole of enzyme.

Free amino groups were determined by reaction of the protein with ninhydrin (Moore and Stein, 1948; Slobodian *et al.*, 1962) using phenylalanine as a standard. The results are expressed as degree of substitution in per cent and were calculated from the difference in ninhydrin values between the control and the modified enzyme. The range for any degree of substitution is of the order of 5%. The values are not corrected for hydrolysis; this correction usually amounts to only 1–2% (Leach, 1966), well within the range of our values.

The stability of the protein was examined in the following manner. The modified enzyme was dialyzed at discrete pH values for 36 hr against buffers adjusted to the desired pH. Changes in conformation or structure owing to alterations in the stability of the protein, as a function of pH, should become apparent over that period of time, as has been shown for Ac_A-carboxypeptidase (Bethune *et al.*, 1964). Subsequent to dialysis the enzyme was assayed immediately for activity under the standard conditions at pH 7.5. This assay would be expected to reflect the irreversible effect of pH on protein stability as opposed to its reversible effect upon the hydrolytic process. The enzyme solutions were then dialyzed for a further 16 hr and assayed again, to ensure that complete deacylation of any tyrosyl residues had occurred. Further deacylation would be manifested as additional activity changes, which were not observed. Physical measurements were performed at the pH of dialysis. The graphic presentation of the results will be referred to as *pH–stability profiles.*

Protein concentrations were determined from the absorbance at 278 mμ using molar absorptivities for native and succinylcarboxypeptidase of 6.32 and 6.47×10^4 l. $mole^{-1}\ cm^{-1}$, respectively, as determined gravimetrically, after 10% trichloracetic acid precipitation followed by drying at 104° (Hoch and Vallee, 1953). A Beckman Model DU spectrophotometer was used throughout. pH was determined with a Radiometer pH m 22 pH meter equipped with a general purpose combined electrode. Where necessary, precautions to prevent contamination by adventitious metal ions were taken throughout these studies (Thiers, 1957).

Modification of the enzyme was carried out routinely by addition of succinic anhydride at a ratio of 48 moles/mole of enzyme in 2 M NaCl–0.02 M Veronal (pH 7.5) at 0° (Riordan and Vallee, 1964). The pH was maintained at 7.5° in a pH-Stat by addition of 1 M NaOH. For certain electrophoretic experiments, a 36 moles/mole ratio was employed to obtain a less extensively modified enzyme. The reaction was carried out for 30 min, after which no further base uptake occurred, and the modified enzyme was then dialyzed until desuccinylated (*vide supra*). Modification of the enzyme with acetic anhydride or acetylimidazole has been described (Bethune *et al.*, 1964).

Electrophoresis in a Spinco Model H electrophoresis and diffusion apparatus was carried out at 3°, at protein concentrations of 0.2–1 g/dl, in a field of 2–3 v/cm. The masks which fit over the electrophoresis cells were modified to incorporate two sets of reference slits to facilitate alignment of the plates in the microcomparator. In addition, a length of fine platinum wire was affixed to one set of the reference slits, forming a reference from which distances are measured.

Mobilities were calculated from the rate of movement of the peak maximum and are reproducible to ±0.1 mobility unit.

Conductivities were determined at 0° with a Radiometer conductivity meter, calibrated with KCl solutions (Longworth, 1959). All mobilities are, therefore, referred to 0°. Heterogeneity constants and diffusion coefficients were determined from plots of $(\sigma_t^2 - \sigma_0^2)/2t$ against t, where σ_0 and σ_t are the half-widths at the inflection points of the schlieren patterns obtained immediately after the current is started and after t seconds, respectively. The slope of the line is $E^2h^2/2$, where E is the field strength in volts per centimeter, h is the heterogeneity constant; the intercept at $t = 0$ (here, the time at which the boundary was sheared) is D, the diffusion coefficient (Alberty *et al.*, 1948). The distribution of mobilities, assumed to be Gaussian, was calculated from the normal error curve for any given value of μ, the mobility, and h.

Sedimentation was carried out in a Spinco Model E ultracentrifuge as described previously (Bethune, 1965). Plates were measured in a two-dimensional comparator (David Mann, Inc.) equipped with a projection screen.

Optical rotatory dispersion (ORD) was measured by means of a Model 200 S-80Q photoelectric spectropolar-

imeter (O. C. Rudolph and Sons) as described previously (Bethune *et al.*, 1964). Measurements of rotatory dispersion at wavelengths shorter than 315 mμ were performed in a Cary Model 60 recording spectropolarimeter at a temperature of 25°. Cells of 0.2–1.0-mm path length with fused-quartz end plates were employed using protein concentrations of 0.4–4 mg/ml. The appropriate dialysates were used as blanks. The slit width of the instrument was programmed to yield maximal and constant light intensities at all wavelengths. Specific rotations were calculated on the basis of protein concentration and are not corrected for the refractive index of the solvent employed.

Viscosities were measured in a Cannon-Fenske viscometer (flow time for water at 20° was 60 sec), reproducibly clamped in a rigid, leveled brass frame, permanently attached to the inner wall of a double-wall water bath and maintained within ±0.002° of the temperature desired by a thermistor-activated heating system. Temperatures were determined by a National Bureau of Standards calibrated thermometer. The kinetic energy correction factor was calculated from measurements of water at 20 and 24°, and amounted to approximately 1%.

Densities were measured at 20° in a dual capillary pycnometer (Fox, 1955) with a capacity of approximately 10 ml. Liquid heights in the capillaries were determined utilizing a cathetometer.

Results[3]

Over the pH range 5–8.5 electrophoresis of *N*-succinylcarboxypeptidase reveals a single boundary (Figure 1), similar to the unmodified enzyme or to Ac_I-carboxypeptidase, but strikingly different from Ac_A-carboxypeptidase, where two boundaries are found (Bethune *et al.*, 1964).

Table I indicates the effect of chemical modification upon the electrophoretic mobility of carboxypeptidase. As would be expected at pH 7.5, Ac_I-carboxypeptidase, in which the free tyrosyl residues alone are modified, exhibits a minimal change in mobility compared to the unmodified enzyme. Mobility is increased in Ac_A-carboxypeptidase, where positively charged free amino groups are converted to uncharged acetylamido groups. The increase in mobility is maximal for the *N*-succinyl enzymes, in which amino groups are converted to negatively charged succinylamido groups.

The mobility of *N*-succinylcarboxypeptidase is a function of the extent of succinylation. This is apparent when mobility is examined for two different degrees of substitution at a single pH (Table I) or as a function of pH (Table II); an increase in the degree of substitution results in increased mobility.

Analysis of the patterns shown in Figure 1 for heterogeneity with respect to mobility (Alberty *et al.*, 1948) yields a heterogeneity constant of 0.29×10^{-5} at pH 7.5 (Figure 2A). The calculated mobility distribution curve shows that 98% of the protein is contained within two mobility units of the average (Figure 2B). When the succinylated protein is examined at other pH values, similar results are obtained. Thus, at pH 5, $h = 0.2 \times 10^{-5}$ and at pH 8.5, $h = 0.38 \times 10^{-5}$. The diffusion coefficient, obtained by extrapolation to zero time, is 5.7×10^{-7} cm²/sec at pH 7.5, 3°, in 0.18 M NaCl–0.02 M sodium barbital buffer, or 9.2×10^{-7} cm²/sec when corrected to water at 20°.

Sedimentation of succinylcarboxypeptidase reveals only a single boundary (Figure 3). The sedimentation process does not depend significantly either on ionic strength or on ion species present (Figure 4). The combined primary and secondary charge effect differences

TABLE I: Electrophoretic Mobility of Native and Chemically Modified Carboxypeptidases at pH 7.5 in 0.18 M NaCl–0.02 M Sodium Barbital Buffer.

Enzyme	$-\mu \times 10^5$ (cm²/v sec) (±0.1)	Deg of Substitution (%) (±5%)
[(CPD)Zn]	1.1[a]	0
[(Ac_ICPD)Zn]	1.3[a]	0
[(Ac_ACPD)Zn]	3.3[b]	48
	3.7[b]	58
[(*N*-SuccinylCPD)Zn]	6.6	61
	8.1	73

[a] NaCl (0.25 M)–sodium barbital (0.02 M) buffer. [b] Refers to the boundary representing undenatured protein (Bethune *et al.*, 1964).

TABLE II: Electrophoretic Mobility of *N*-Succinylcarboxypeptidase and the Effect of pH and Extent of Modification.[a]

Deg of Substitution (%)	pH	$-\mu \times 10^5$ (cm²/v sec)
73	5.5	7.6, 7.5
73	6.8	7.6
73	7.5	8.2, 8.1
69	8.5	8.4
59	5.0	5.5, 5.6
61	6.0	5.7
61	7.5	6.5, 6.6
51	8.0	8.5

[a] Buffers for the different pH ranges are as in Figure 1.

[3] Preliminary reports (Bethune and Ulmer, 1963; Vallee, 1964) of some of the results are here recalculated based on experimental determination of the extinction coefficients of the various derivatives.

FIGURE 1. Electrophoretic patterns of *N*-succinylcarboxypeptidase at different pH values. In each instance the cathode and the descending boundary are on the left. The break in the base line signifies the superposition of photographs of the ascending and descending boundaries, taken within 60 sec of each other. Conditions: sodium acetate (0.02 M)–sodium chloride (0.18 M) (pH 5.0), after 4248 sec at 2.35 v/cm; sodium barbital (0.02 M)–sodium chloride (0.18 M) (pH 7.5), after 9000 sec at 2.67 v/cm; sodium barbital (0.02 M)–sodium chloride (0.18 M) (pH 8.5), after 7320 sec at 2.28 v/cm. All times refer to the descending boundary.

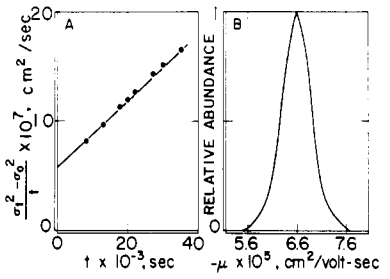

FIGURE 2. Determination of the heterogeneity constant, *h*, and diffusion coefficient, *D*, for *N*-succinylcarboxypeptidase (A) and calculated mobility distribution (B). (A) At 3° and pH 7.5 in sodium barbital (0.02 M)–NaCl (0.18 M) buffer, degree of substitution, 61%.

subsequent to a change in ionic strength from 0.18 to 2 M are calculated (Pedersen, 1958) to be of the order of 0.01 S. The difference in secondary charge effect between sodium and potassium chlorides is of the order of 0.001 S, specific ion effects being absent. Both effects are outside the limits of detection of sedimentation velocity experiments under the conditions employed.

The sedimentation values extrapolate to an $s^0_{20,w}$ of 3.55 S and exhibit negative concentration dependence. In contrast, the concentration dependence of the native enzyme is positive over this concentration range (Rupley and Neurath, 1960; Bethune, 1965).

Viscometric studies of the modified and native enzymes over a concentration range of 6–20 mg/ml reveal no gross changes with respect to shape, yielding limiting values of the intrinsic viscosity of 3.5 and 3.4 ± 0.2 ml/g for the *N*-succinyl and native enzymes, respectively.[4]

[4] These values were obtained in 1 M NaCl–0.02 M sodium barbital (pH 7.5). *N*-Succinylcarboxypeptidase was also examined in 0.18 M NaCl–0.02 M sodium barbital (pH 7.5) and yielded the same limiting intrinsic viscosity.

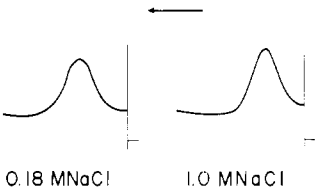

FIGURE 3. Sedimentation patterns of *N*-succinylcarboxypeptidase, 60% degree of substitution. Direction of motion is from right to left. (A) After 3600 sec at 59,780 rpm, 20°. In 0.18 M NaCl–0.02 M sodium barbital (pH 7.5); enzyme, 11.7 mg/ml. (B) After 4200 sec at 59,780 rpm, 22°. In 1 M NaCl–0.02 M sodium barbital, (pH 7.5); enzyme, 12.8 mg/ml.

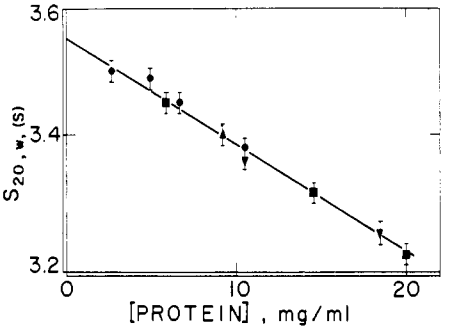

FIGURE 4. Concentration dependence of the sedimentation coefficient of *N*-succinylcarboxypeptidase (pH 7.5), 0.02 M sodium barbital. (●) 0.18 M KCl, (■) 0.18 M NaCl, (▲) 1.0 M NaCl, and (▼) 2.0 M NaCl.

The pH–stability profiles of both the esterase activity and of ORD parameters (Figure 5) of *N*-succinylcarboxypeptidase reveal but minimal changes as a function of pH. In this respect *N*-succinylcarboxypeptidase resembles the native and Ac_I enzymes but again differs from Ac_A-carboxypeptidase, where the values at higher pH were consistent with denaturation (Bethune *et al.*, 1964). The values at pH 7.5 of the relevant ORD parameters for the *N*-succinyl and native enzymes are compared in Table III. Succinylation neither alters the magnitude of the trough of the intrinsic Cotton effect at

TABLE III: ORD Parameters of Native and *N*-Succinylcarboxypeptidase at pH 7.5 in 1 M NaCl–0.02 M Sodium Barbital Buffer.

	Native	Succinyl
$[\alpha]^{10}_{546}$, deg	−26 ± 2	−26 ± 2
$[\alpha]^{25}_{235}$, deg	−4200 ± 200[a]	−4300 ± 200[a]
λ_c, mμ	263 ± 3	267 ± 3
b_0	−146 ± 10	−143 ± 10

[a] 1 M NaCl–0.05 M Tris (pH 7.5).

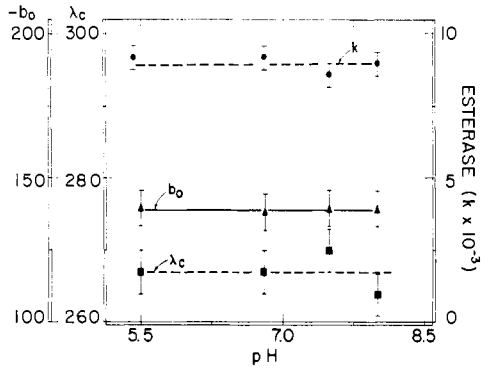

FIGURE 5. pH–stability profiles of esterase (k) activity, λ_0 and b_0 of *N*-succinylcarboxypeptidase. The buffers used were as in Figure 1.

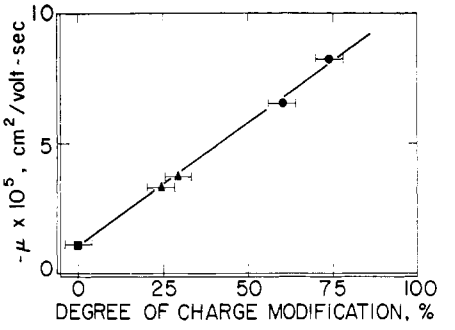

FIGURE 6. Mobility of native (■), *N*-succinyl- (●), and Ac_A- (▲) carboxypeptidase as a function of degree of substitution in per cent. The data are from Table I. The degree of substitution for the Ac_A enzymes is divided by two before plotting (see text).

235 mμ nor does it affect the shape of the side-chain perturbations in the spectral range 270–290 mμ. Similarly, the values of the dispersion constant, λ_0, and of b_0, of the modified enzyme, are not significantly different from those of native carboxypeptidase, denoting minimal structural change. The peak of the intrinsic Cotton effect could not readily be evaluated due to the marked solvent absorption below 215 mμ.

Discussion

Chemical modifications with site-specific selective reagents have proven an important approach in identifying amino acid side chains critical to the catalytic function of enzymes. However, in interpreting the results of any particular modification, it is recognized that enzymatic activity might be affected through several possible mechanisms. Activity can be altered *directly* through modification of a specific side chain at the "active center" of the enzyme or *indirectly* as the result of changes in protein secondary, tertiary, or quaternary structure. Both direct and indirect effects may operate simultaneously. Catalytic function can also be altered, to a minor degree, if the modification affects a general property of the protein such as charge, as demonstrated here. The relative significance of each of these effects may vary considerably when the same reagent is employed to modify different enzymes, or when similar reagents are employed with the same enzyme. Valid conclusions as to the functional role of amino acid side chains based upon the results of chemical modifications must, therefore, derive both from evaluation of enzymatic activities and from analysis of the physicochemical properties of the modified proteins.

We have shown previously that simultaneous acetylation of both tyrosyl and lysyl residues changes the physicochemical properties of carboxypeptidase. The ORD of Ac_A-carboxypeptidase indicates partial denaturation, and electrophoresis reveals the presence of an inactive component (Bethune *et al.*, 1964). In contrast, the optical rotatory or electrophoretic properties of Ac_I-carboxypeptidase, where only tyrosyl residues are acetylated, do not differ from those of the nat ve enzyme. The spontaneous desuccinylation of *N,O*-succinylcarboxypeptidase to form *N*-succinylcarboxypeptidase (Riordan and Vallee, 1964) provides an opportunity to investigate the physical chemistry of a derivative of this enzyme in which lysyl, but not tyrosyl, residues are modified extensively.

Electrophoresis of *N*-succinylcarboxypeptidase over the pH range 5.0–8.5 reveals only a single boundary (Figure 1). Moreover, examination of the patterns for heterogeneity (Alberty *et al.*, 1948) indicates a remarkably small range of mobility values (Figure 3). The chemically modified enzyme is insoluble below pH 5.0; thus, this method cannot be employed at the average isoelectric point where complicating effects due to pH and conductivity charges are minimized. Nevertheless, it has proven to be of value even at other pH values (Alberty *et al.*, 1948). Calculations were performed here only for the descending boundary, where pH and conductivity changes may cause spreading in addition to that resulting from diffusion and heterogeneity alone. Indeed, the ascending boundary pattern is sharper than that of the descending one, and buffer concentration boundaries are present as would be expected when these effects obtain (Figure 1); moreover, as the isoelectric point is approached the heterogeneity constant becomes somewhat smaller. The results, therefore, reflect only the *upper* limit of the distribution of mobility values; the true distribution may indeed be narrower than indicated. The mobility distribution plot at pH 7.5 indicates that less than 1% of the protein has a mobility differing by two units from that of the average, determined from the rate of movement of the peak maximum (Figure 2B). Since the mobilities of the two *N*-succinyl enzymes differ by 1.5 mobility units (Table I), their mobility distributions overlap only to a very small extent, suggesting that succinylation occurs as a stepwise rather than a random reaction. This is attributable to differential reactivity of the groups undergoing succinylation.

Moreover, the mobilities of the *N*-succinyl and Ac_A-carboxypeptidases at pH 7.5 are linearly related to the degree of substitution, as measured by the ninhydrin reaction (Figure 6). For this comparison the degree of substitution for the Ac_A enzymes, shown in Table I, is divided by two, since, at pH 7.5, succinylation changes the charge of an amino group from +1 to −1 while acetylation changes it only from +1 to 0 (changes in ion-binding and neighboring group effects are here neglected). The relationship exhibited in Figure 6 is, therefore, between mobility and *charge* modification. All these derivatives exhibit the same linear relationship of mobility to measured charge modification. This suggests that changes in mobility are due entirely to modification of amino groups and supports the conclusion that the observed narrow mobility distribution is due to a differential degree of reactivity of such groups with succinic anhydride.[5]

It is well recognized that amino acid residues of native proteins can be divided into classes depending upon the spectrum of reactivities they display toward various chemical agents. The different classes of tyrosyl residues (Wetlaufer, 1962) and sulfhydryl groups (Cecil and McPhee, 1959) are, perhaps, best delineated in this regard: with a given reagent one set reacts readily while another reacts much less promptly. Based on their reaction with succinic anhydride, the amino groups of carboxypeptidase also seem to fall into two categories. Even at a 96-fold molar excess of the reagent, approximately four free amino groups (measured as phenylalanine equivalents) remain unmodified (Riordan and Vallee (1964); see Table I). The likely differential reactivity of those amino groups which are readily susceptible to modification, inferred here from electrophoretic measurements,[6] suggests further subdivisions, as has been postulated for the interaction of tyrosyl residues of ribonuclease with iodine (Friedman *et al.*, 1966).

As with the native enzyme, sedimentation of *N*-succinylcarboxypeptidase reveals only a single boundary (Bethune, 1965). However, at protein concentrations up to 20 mg/ml, the sedimentation coefficient of the native enzyme exhibits a positive dependence on concentration, indicating the presence of a polymer (Rupley and Neurath, 1960; Bethune, 1965). In contrast, the concentration dependence of the sedimentation coefficient of *N*-succinylcarboxypeptidase is negative. Presumably the increased charge on *N*-succinylcarboxypeptidase is sufficient to prevent the rather weak interaction occurring in solutions of the native enzyme.

The extrapolated sedimentation coefficient is 3.55 S. When combined with the diffusion coefficient, determined from analysis of the electrophoretic patterns, and a partial specific volume of 0.73 ml/g, calculated from amino acid analysis (Bargetzi *et al.*, 1963), a molecular weight of 35,000 is obtained. The molecular weight of the native enzyme, calculated from amino acid analysis, is 33,800 ± 300 (Bargetzi *et al.*, 1963). The addition of nine succinyl groups (representing a degree of modification of 60%) would increase the molecular weight by 900, yielding a total molecular weight of 34,700 ± 300 for succinylcarboxypeptidase, in excellent agreement with the measured value.

The limiting value of the intrinsic viscosity for *N*-succinylcarboxypeptidase is identical with that of the native enzyme, within the error of measurement, demonstrating that no gross change in shape has occurred as a result of chemical modification. The value obtained, 3.3–3.7 ml/g, is well within the range found for globular proteins.

Thus, the results obtained from the three transport methods, electrophoresis, sedimentation, and viscosity, demonstrate no gross heterogeneity with respect to charge or mass, and, at most, only minor changes with respect to shape.

ORD is generally considered to be dependent upon

[5] If reaction occurs randomly, then the distribution of reacted groups is predictable, given some measure of the average degree of substitution (Baldwin *et al.*, 1951; Fisher and Lauffer, 1949). Under these conditions the standard deviation of the binomial distribution of reacted groups, β, becomes $\sqrt{Sq(1-q)}$, where S is the total number of modifiable residues, and q is the measured fractional degree of substitution. The standard deviation of the *mobility* distribution is then $k\beta$, where k is the mobility increment per residue modified. There are 15 lysyl residues in carboxypeptidase in terms of ninhydrin-reactive groups. With this value for S, together with the data in Table I, $\beta = 1.8$ residues (being rather insensitive to the value of S); the measured value of h, 2.9×10^{-1} mobility unit, then yields an estimate for k of 0.16 mobility unit/residue modified. A direct estimate of k is obtained from the mobilities of the different modified enzymes at pH 7.5 and is approximately 1 mobility unit/residue modified. This discrepancy can be explained if the residues are not equally reactive toward the reagent or if more residues than those detected by the ninhydrin reaction are modified, or both. That the second possibility is less probable is demonstrated in Figure 6, and is also inferred from consideration of the likely reactivity of various residues in the protein with succinic anhydride. Thus, the reactivity of the α-amino group of carboxypeptidase is markedly lowered in the holoenzyme (Coombs *et al.*, 1964) and would presumably behave as a poorer nucleophile than would be anticipated for an isolated amino group; the ϵ-amino groups of lysine react, as demonstrated by reaction with ninhydrin; tyrosyl residues are modified (Riordan and Vallee, 1964), but upon prolonged dialysis spontaneous desuccinylation occurs. Succinylhistidine is extremely labile (Greenstein and Winitz, 1961), and, if formed, would hydrolyze virtually instantaneously. Lastly, the hydroxyl groups of serine and threonine are extremely poor nucleophiles compared with free hydroxyl ions and amino groups, and it would appear reasonable, *a priori*, to exclude their general modification. Moreover, in contrast to many other hydrolytic enzymes, carboxypeptidase does not contain serine groups of exceptional reactivity, demonstrated by the lack of reaction with diisopropyl fluorophosphate (Brown *et al.*, 1963).

The formation of the ϵ-*N*-succinimide derivative of lysine, found in succinyl subtilopeptidase (Gounaris and Ottesen, 1965), would be rendered less probable in view of the data in Figure 6, since the compound produces the same color yield with ninhydrin as does lysine (Gounaris and Ottesen, 1965), but changes the charge of the amino group only from +1 to 0.

[6] Although the specificity of the reagent has not been a primary objective of this study, these conclusions could be susceptible to direct chemical confirmation by reaction of the succinylated enzyme with, *e.g.*, a diazo reagent, such as diazonium-1H-tetrazole, followed by amino acid analysis and by peptide mapping.

the secondary and tertiary structure of the protein molecule. The values of the relevant parameters are coincident with those of the native enzyme within one standard deviation (Table III). Thus, no detectable differences in conformation result from modification of the free amino groups.

These results demonstrate the stability of the carboxypeptidase molecule. The electrostatic repulsion resulting from modification of the charge on the mo ecule, manifested in the increase in electrophoretic mobility, does not lead to structural changes subsequent to electric imbalance, which can result in disruption of the native structure of proteins (Weber and Tanford, 1959). Moreover, succinylation, often employed for dissociation of complex proteins, has no pronounced effect on the limiting value of the sedimentation coefficient, although it does manifest a related effect by preventing polymerization of the enzyme (Bethune, 1965).

As would be anticipated from the results of electrophoresis, the pH stability of the succinyl enzyme resembles closely that of the native enzyme. Variations in any of the parameters measured are essentially absent over this pH range (Figure 5). With respect to catalytic function, the absolute values for both peptidase and esterase activity are slightly greater than those of the native enzyme (Riordan and Vallee, 1964). These minor changes in enzymatic activities, subsequent to reaction, demonstrate that the amino groups attached are not vitally involved in the active center of the enzyme, and emphasize the mildness of the succinylation reaction.

Thus, the investigation of the physicochemical properties of this carboxypeptidase derivative show that only two pertinent properties of the enzyme have been affected by chemical modification, after spontaneous desuccinylation has occurred. The electrophoretic mobility is increased roughly fivefold. The concentration dependence of the sedimentation coefficient changes from positive to negative. In both instances these changes may be explained as the result of the increased charge of the molecule. The reaction of succinic anhydride with amino groups changes the charges on these from positive to negative, resulting in an increased net negative charge on the molecule. Thus, succinylation of carboxypeptidase alters significantly only the charge-dependent physicochemical properties of the molecule. Essentially similar conclusions have been drawn from the study of succinyl subtilisin (Gounaris and Ottesen, 1965).

It has now been possible to investigate three different derivatives of carboxypeptidase A: one with *O*-acetyltyrosyl residues, one with *N*-succinylamido groups, and one containing both *O*-acetyltyrosine and *N*-acetylamido groups. When either tyrosine or lysine alone is modified, no significant stability changes in the structure of the protein molecule have been demonstrated, although the functional consequences of the two modifications differ markedly (Simpson *et al.*, 1963; Riordan and Vallee, 1964). The derivative in which both types of residues have been modified, while exhibiting changes in function similar to those subsequent to *O*-tyrosyl acetylation, displays instability in both structure and catalytic activity as a function of pH.

References

Alberty, R. A., Anderson, E. A., and Williams, J. W. (1948), *J. Phys. Colloid Chem. 52*, 217.

Anson, M. L. (1937), *J. Gen. Physiol. 20*, 663, 777.

Baldwin, R. L., Laughton, P. M., and Alberty, R. A. (1951), *J. Phys. Colloid Chem. 55*, 111.

Bargetzi, J. P., Sampath Kumar, K. S. V., Cox, D. J., Walsh, K. A., and Neurath, H. (1963), *Biochemistry 2*, 1468.

Bethune, J. L. (1965), *Biochemistry 4*, 2691.

Bethune, J. L., and Ulmer, D. D. (1963), *Federation Proc. 22, No. 2*, 594.

Bethune, J. L., Ulmer, D. D., and Vallee, B. L. (1964), *Biochemistry 3*, 1764.

Brown, J. R., Greenshields, R. N., Yamasaki, M., and Neurath, H. (1963), *Biochemistry 2*, 867.

Cecil, R., and McPhee, J. R. (1959), *Advan. Protein Chem. 14*, 256.

Coleman, J. E., and Vallee, B. L. (1960), *J. Biol. Chem. 235*, 390.

Coombs, T. L., Omote, Y., and Vallee, B. L. (1964), *Biochemistry 3*, 653.

Fisher, M. A., and Lauffer, M. A. (1949), *J. Am. Chem. Soc. 71*, 3800.

Fox, J. B., Jr. (1955), Ph.D. Dissertation, University of Washington, Seattle, Wash.

Friedman, M. E., Scheraga, H. A., and Goldberger, R. F. (1966), *Biochemistry 5*, 3770.

Greenstein, J. P., and Winitz, M. (1961), Chemistry of the Amino Acids, New York, N. Y., Wiley, p 2117.

Gounaris, A., and Ottesen, M. (1965), *Compt. Rend. Trav. Lab. Carlsberg 35*, 37.

Hoch, F. L., and Vallee, B. L. (1953), *Anal. Chem. 25*, 317.

Leach, A. A. (1966), *Biochem. J. 98*, 506.

Longworth, L. G. (1959), *in* Electrophoresis, Bier, M., Ed., New York, N. Y., Academic.

Moore, S., and Stein, W. H. (1948), *J. Biol. Chem. 176*, 367.

Pedersen, K. O. (1958), *J. Phys. Chem. 62*, 1282.

Piras, R., and Vallee, B. L. (1966), *Biochemistry 5*, 885.

Riordan, J. F., Sokolovsky, M., and Vallee, B. L. (1966), Abstracts, 152nd National Meeting of the American Chemical Society, Sept 1966, New York, N. Y.

Riordan, J. F., and Vallee, B. L. (1963), *Biochemistry 2*, 1460.

Riordan, J. F., and Vallee, B. L. (1964), *Biochemistry 3*, 1768.

Rupley, J. A., and Neurath, H. (1960), *J. Biol. Chem. 235*, 609.

Simpson, R. T., Riordan, J. F., and Vallee, B. L. (1963), *Biochemistry 2*, 616.

Simpson, R. T., and Vallee, B. L. (1966), *Biochemistry 5*, 1760.

Slobodian, E., Mechanic, G., and Levy, M. (1962), *Science 135*, 441.

Sokolovsky, M., and Vallee, B. L. (1966), Abstracts, 152nd National Meeting of the American Chemical Society, Sept 1966, New York, N. Y.

Sokolovsky, M., and Vallee, B. L. (1967), *Biochemistry 6*, 700.
Thiers, R. E. (1957), *Methods Biochem. Anal. 5*, 273.
Vallee, B. L. (1964), *Federation Proc. 23*, 8.
Weber, R. E., and Tanford, C. (1959), *J. Am. Chem. Soc. 81*, 3255.
Wetlaufer, D. B. (1962), *Advan. Protein Chem. 17*, 303.

Some Physical and Chemical Studies on the Protein Moiety of a High-Density (1.126–1.195 g/ml) Lipoprotein Fraction of Human Serum*

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ABSTRACT: The protein moiety of a high-density (1.126–1.195 g/ml or HDL_3) lipoprotein fraction from human serum was obtained in greater than 95% yield in lipid-free, water-soluble form after removal of the lipids. Dissociation of the protein into subunits was facilitated by sodium dodecyl sulfate. Sedimentation equilibrium experiments with HDL_3 protein were conducted principally in systems consisting of protein, detergent, and buffer under conditions of essentially complete binding of the detergent by protein. These experiments indicated homogeneity with respect to the size of protein subunits and a molecular weight between 30 and 31 $\times 10^3$ for the subunit. The molecular weight calculated from the amino acid composition is in good agreement with this value. From the previously reported molecular weight and the per cent protein of the lipoprotein, it was concluded that there are three protein subunits per molecule in the intact lipoprotein.

The high-density (1.065–1.20 g/ml) lipoprotein fraction isolated from human serum shows in the analytical ultracentrifuge two major components, which have been designated HDL_2 and HDL_3 (DeLalla and Gofman, 1954). The lipoproteins of density 1.125–1.21 g/ml, or HDL_3, contain about 53% (present work) to 57% (Scanu and Granda, 1966) protein. From the reported molecular weight values of 175,000 (Hazelwood, 1958) and 170,000 (Scanu and Granda, 1966) for HDL_3 and the per cent protein, the protein moiety can be estimated to be about 95,000 g/mole of lipoprotein. Although the intact lipoprotein has not been dissociated into subunits, apparently the protein moiety is comprised of subunits whose dissociation in the absence of lipid is favored by the presence of detergent. After removal of lipids, the protein moiety in aqueous solution was found by Scanu *et al.* (1958) to have a molecular weight of 75,000, and by Shore and Shore (1962) to be comprised of a major and a minor component with $s_{20,w}$ values 2.3–2.6 and 4.2–4.6, respectively, as well as some highly aggregated material. On addition of SDS[1] to the protein solution, subunits of mol wt 36,000 (Shore and Shore, 1962) and 21,500 (Scanu and Granda, 1966) were found by the Archibald (1947) method of approach to sedimentation equilibrium.

In the present study, sedimentation equilibrium was used to obtain information on the molecular weight and homogeneity of the subunits of the protein moiety of density 1.125–1.195-g/ml lipoproteins of human serum. A complete amino acid analysis of the protein is also presented.

Materials and Methods

Lipoprotein Isolation. Lipoproteins of density 1.126–1.195 g/ml were isolated from human serum from individual donors, both male and female. Aliquots of 0.2 M Na-EDTA at pH 7.4 were added as needed to maintain a concentration of 8×10^{-4} M during all stages of isolation of the lipoprotein fraction. Less dense lipoproteins were removed by two centrifugations at a solvent density of 1.124 g/ml (20°). In the first, serum adjusted to this density with solid sodium chloride was centrifuged 48 hr at 39,000 rpm in a 40.3 rotor at 13–14° in a Spinco Model L centrifuge. The bottom 3-ml portions in the tubes were combined, diluted with an equal volume of salt solution of density 1.124 g/ml, and centrifuged as above. After the second centrifugation, the bottom 3-ml portions in the tubes were combined and adjusted to a solvent density of 1.195 g/ml by addition of a D_2O solution of sodium nitrate of density 1.450 and pH 7.4. The resulting

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[1] Abbreviation used: SDS, sodium dodecyl sulfate.