Journal of Bioenergetics and Biomembranes (1977) **9**, 181–193

Succinylated Bovine Heart Mitochondrial Cytochrome *c* Oxidase

Kathleen Hillman* and W. W. Wainio

Department of Biochemistry and the Bureau of Biological Research,
Rutgers College, Rutgers University
The State University of New Jersey,
New Brunswick, New Jersey 08903

Received 2 February 1977

Abstract

Cytochrome *c* oxidase was prepared by sequential extraction of bovine heart muscle submitochondrial particles with sodium deoxycholate, followed by fractional precipitation with ammonium sulfate and chromatography on Sephadex G-75. The resulting preparation had typical absorption spectra, an activity of 1.28 sec^{-1} (mg protein)$^{-1}$ (3 ml)$^{-1}$ in deoxycholate or 4.13 sec^{-1} (mg protein)$^{-1}$ (3 ml)$^{-1}$ in 0.5% Tween 80, and a minimum molecular weight of 120,000 daltons as calculated from the heme content and the total protein. Amino acid analyses of nine preparations yielded a molecular weight per heme of 86,500 daltons. The net charge was calculated to be +8.7 at pH 7.0. Succinylation of cytochrome *c* oxidase in the presence of 500 molar excess of succinic anhydride produced a soluble preparation having a negative charge at neutral pH. The modified enzyme was highly autoxidizable and had little or no activity toward ferrocytochrome *c* as a substrate. Its average $S_{20,w}$ was 5.8 and its apparent D was 4.0×10^{-7} $cm^2\ sec^{-1}$, from which a molecular weight of 126,000 daltons was calculated. This size of enzyme is considered to be that of the monomer, because the value is practically the same as the minimum molecular weight reported herein, and since it is approximately one-half the value obtained in our laboratory (and in others) for the unmodified enzyme.

* Recipient of a National Science Foundation predoctoral fellowship. Present address: M.D. Anderson Hospital and Tumor Institute, Texas Medical Center, Houston, Texas 77025.

This journal is copyrighted by Plenum. Each article is available for $7.50 from Plenum Publishing Corporation, 227 West 17th Street, New York, N.Y. 10011.

Introduction

Succinylation has been used to place a negative charge on proteins, to solubilize proteins, and to introduce functional groups into proteins [1]. The reaction of succinic anhydride with the side chains of proteins is primarily with the lysyl residues, especially since the reaction products with other amino acid residues are highly unstable [2]. Examples of dissociation of proteins by succinylation are found in the work of Klotz and Keresztes-Nagy [3] and Habeeb et al. [4]. Extensive modification of an enzyme can be accomplished without serious loss of activity, as demonstrated by Gounaris and Ottesen [2] with subtilopeptidase.

With respect to the respiratory chain, MacLennan et al. [5] used succinylation to confer water solubility on a number of mitochondrial proteins. They were able to separate nonheme iron proteins of complex III from the cytochromes *b* and c_1 contained in that segment of the chain. Complex IV (cytochrome *c* oxidase) when succinylated yielded a high-copper, low-heme protein of molecular weight 25,000 [6]. Approximately two atoms of copper were associated with each 25,000 g of protein, but since the EPR spectrum was altered, they concluded that some, if not all, of the copper had been translocated.

Minimum molecular weights (per heme) for cytochrome oxidase range from 67,000 [7] to 124,000 [8]. Since the intact enzyme has a molecular weight of about 200,000 [9] as determined by the sedimentation velocity method, it would be expected that the monomer, if the two parts of the dimer were equal, should have a molecular weight of about 100,000 [9].

The approximate value of 100,000 for the minimum molecular weight is supported by amino acid analyses of the enzyme. Matsubara et al. [10] reported a protein molecular weight of 93,378, whereas Wainio et al. [11] found a value of approximately 85,000 for one preparation.

Methods

Submitochondrial particles from bovine heart were prepared by a modification [12] of the original method Keilin and Hartree [13]. The final precipitate of submitochondrial particles was adjusted to 25 mg protein/ml with 0.1 M phosphate buffer, pH 7.1.

Cytochrome oxidase was prepared from the particles as follows: Sodium deoxycholate was added to a concentration of 1.75% (17.5 mg/ml) and the suspension was centrifuged for 45 min at 25,000 rpm in the No. 30 head of the Beckman Model L ultracentrifuge. The dark green pellets were suspended by homogenization in a volume of 0.1 M phosphate buffer, pH 8.3, equal to the volume of the submitochondrial suspension originally

taken. The suspension was centrifuged at 25,000 × *g* for 15 min. The pellets were resuspended in the same volume of phosphate buffer, pH 8.3. Sodium deoxycholate was added to a concentration of 2.0% (20 mg/ml) and the suspension was centrifuged at 25,000 × *g* for 30 min. The pellets were discarded. The supernatant fluid was diluted with 4 volumes of 0.1 M phosphate buffer, pH 8.3, at room temperature. A solution of $(NH_4)_2SO_4$ saturated at room temperature was added slowly with stirring to 10% saturation (v/v). The pH was maintained between 8.0 and 8.3 by the simultaneous addition of 0.5 N NaOH. The cloudy solution was centrifuged at 25,000 × *g* for 30 min. The pellets were discarded. The supernatant fluid was treated with saturated $(NH_4)_2SO_4$ to 30% (v/v) and the pH was maintained between 8.0 and 8.3 with NaOH. The cloudy solution was centrifuged at 25,000 × *g* for 30 min. The supernatant fluid was discarded.

The dark green gelatinous pellets were washed three times with distilled water without disruption. The pellets were dissolved in one-third the original volume of 0.1 M phosphate buffer, pH 8.3, or in the same amount of buffer containing 0.5% (5 mg/ml) of Tween 80. The Tween 80 was necessary only if the enzyme was to be stored overnight in the cold room. However, it is necessary that the enzyme be chromatographed immediately so that it will not lose activity.

Sephadex G-75 which had been equilibrated with 0.5% Tween in 0.1 M phosphate buffer, pH 8.3, for at least 12 hr was used to prepare a column 4 cm in diameter and 57 cm in height (approximately 200 ml of wet Sephadex). From 10 to 15 ml of the oxidase in buffer was placed on the column at room temperature and the flow rate was adjusted to between 0.5 and 0.75 ml/min. The oxidase moved as a single band which left the column at the void volume—usually between 70 and 80 ml. The volume of enzyme collected was usually no greater than twice the volume applied and contained from 3 to 4 mg protein/ml. When more dilute solutions were obtained they were concentrated by dialysis against 20 or 40% Carbowax dissolved in the elution buffer. *Protein* was determined by the Biuret method slightly modified. A solution containing all the reactants, except the copper, was used to correct for the absorbance by the enzyme at 500 nm. All spectra were recorded with a Cary 11 spectrophotometer. *Heme* was determined from the absorbance difference of the enzyme at 602.5 nm (reduced minus oxidized, $\epsilon = 0.76 \times 10^5$). *Deoxycholate* was determined by the method of Szalkowski and Mader [14] as adapted by Kremzner and Wainio [15], and where greater sensitivity was required, the oxidase solution was concentrated three-fold against Carbowax (w/v) and the 1 : 4 dilution was omitted.

Amino acid analyses were performed with a Durrum 500 Analyzer. The

rate of destruction of the amino acids was determined by hydrolyzing one preparation with HCl for 18, 42, and 66 hr and extrapolating to zero time. The average values for nine preparations were corrected with these factors. The largest corrections were for threonine (+17%) and serine (+30%). All other corrections were less than +7.5%. Tryptophan was determined by the method of either Opieńska-Blauth et al. [16] or Spies [17].

Gel electrophoresis was on a 5% acrylamide system. The gels and buffers were made according to Davis [18] as described by Maurer [19]. The time was 1.5 hr, the current 3 mA per tube, and the stain Coomassie Blue.

Sedimentation coefficients and apparent diffusion constants were determined at 20°C with a Beckman Model E ultracentrifuge. The speed was 59,780 rpm for sedimentation and 10,531 rpm for diffusion. The partial specific volume was assumed to be 0.72 [20, 9].

The activity of cytochrome oxidase was determined spectrophotometrically at 25°C by following the oxidation of ferrocytochrome *c* (Sigma Chemical Company, Type VI, equine heart) at 550 nm in 0.1 M phosphate buffer, pH 6.0 [21].

The degree of modification by succinylation was determined by further guanidinylation with *O*-methylisourea. Since the homoarginyl residues formed from the lysyl residues with *O*-methylisourea are stable to acid hydrolysis, whereas the succinylated lysyl residues are not, all the lysine which appears in the amino acid analysis can be said to have been succinylated. Guanidinylation which was more than 95% complete when nonsuccinylated oxidase was used was accomplished by adding urea to 8 M and *O*-methylisourea to 1 M to a solution of succinylated oxidase containing between 6 and 10 mg protein. The pH was adjusted to between 10.3 and 10.5 and the reaction mixture was placed in a cold room at 4°C for 5 days. The protein was precipitated with 30% trichloroacetic acid (w/v). The precipitate after centrifugation was washed several times with 95% ethanol and once with ethyl ether, and was dried under vacuum.

Results

Thirteen preparations of unmodified cytochrome oxidase had an average value of 602.5 nm for the position of the α peak in the reduced state. The γ peak was at 443 nm in the reduced state and at 424 nm in the oxidized state. The ratios (reduced γ)/(oxidized γ) and (reduced γ)/(reduced α) were 1.20 and 4.75, respectively. The heme/protein ratio averaged 0.60 based on an assumed molecular weight of 72,000, and 0.82 based on an assumed molecular weight of 100,000. The minimum protein molecular weight calculated from the heme content was therefore 120,000. The specific activity for the oxidation of ferrocytochrome *c* in air averaged 1.28 sec^{-1}

(mg protein)$^{-1}$ (3 ml)$^{-1}$ when the enzyme was in deoxycholate (eight preparations) and 4.13 sec^{-1} (mg protein)$^{-1}$ (3 ml)$^{-1}$ when the deoxycholate was replaced with 0.5% Tween 80 (three preparations).

One preparation had a (reduced γ)/(oxidized γ) peak ratio of 1.38 and a (reduced γ)/(reduced α) peak ratio of 4.88. The minimum molecular weight was 100,000 and the activity of the enzyme in Tween 80 was 4.78 sec^{-1} (mg protein)$^{-1}$ (3 ml)$^{-1}$.

Nine preparations of unmodified cytochrome oxidase were analyzed for their amino acid content. All preparations except 24 (30 hr) and 29 (42 hr) were hydrolyzed for 36 hr. The results are presented in Table I. Methionine was calculated from methionine plus methionine sulfoxide. The average values are presented in Table II where the analyses of Matsubara et al. [10] and Wainio et al. [11] are presented for comparison. There are differences, but none of these can be explained, except perhaps on the basis of impurities that all preparations must contain.

An estimate of the charge carried by the protein at several pH values was made by employing the pK_a values of the individual amino acids (Table III). The number of aspartyl and glutamyl residues present as the

TABLE I. Amino Acid Analyses of Unmodified Cytochrome *c* Oxidase Preparations

	Preparation no.								
Amino acid	10	11	12	13	14	17	20	24	29
Asp	54.4[a]	59.9	57.1	57.3	55.1	61.5	60.8	53.9	56.2
Thr	43.6	51.2	43.7	48.0	39.5	37.4	42.6	41.5	39.2
Ser	30.3	45.2	38.1	40.7	30.4	43.4	44.3	38.3	30.7
Glu	59.1	60.3	60.1	62.2	57.4	67.2	64.6	[b]	64.2
Pro	37.5	39.3	34.7	[c]	33.1	[c]	[c]	37.0	[c]
Gly	48.3	52.0	51.5	52.0	50.3	51.0	53.8	52.0	50.9
Ala	55.9	55.6	56.1	55.6	54.4	56.5	58.0	55.6	51.0
Val	46.1	40.0	43.1	42.6	45.0	41.7	43.0	44.5	43.5
Met	[d]	[d]	24.0	[d]	24.9	20.9	22.8	[c]	26.6
Ile	37.3	35.5	37.7	37.3	37.6	34.5	35.8	37.3	37.4
Leu	68.7	73.1	70.9	72.7	68.9	69.5	72.9	72.8	73.9
Tyr	21.9	25.1	21.5	22.5	24.7	26.4	27.0	23.7	27.1
Phe	38.6	39.4	37.7	37.5	36.2	35.5	36.7	38.6	39.8
His	19.9	22.4	18.9	21.2	19.9	19.4	19.7	21.2	22.8
Lys	36.4	38.4	36.7	39.4	36.5	41.9	37.6	35.9	37.2
Arg	28.9	31.0	28.1	28.1	31.2	31.4	28.8	28.4	28.7

[a] Expressed in moles per mole of heme.
[b] Machine error.
[c] Hydrolysate not adjusted to pH 7.0.
[d] Temperature effect: MSO merged with Asp.

TABLE II. Comparison of Amino Acid Compositions of Three Different Preparations of Cytochrome *c* Oxidase

Amino acid	This paper	Matsubara et al. [10]	Wainio et al. [11]
Asp	61	60	55
Thr	50	53	42
Ser	49	54	35
Glu	64	60	53
Pro	36	46	32
Gly	53	59	53
Ala	56	62	56
Val	44	51	41
Met	24	32	22
Ile	37	43	37
Leu	73	87	76
Tyr	26	33	27
Phe	38	47	43
His	21	30	20
Lys	39	39	42
Arg	31	31	27
Cys	–	6	5
Trp	29	30	44
NH_3	(62)[a]	(59)[a]	(111)[a]
Molecular weight	83,967[b]	94,399[c]	84,022

[a] Excluded from the molecular weight.
[b] When heme, NH_3, and Cys are added, the value is 86,477.
[c] Recalculated for the average of two preparations.

TABLE III. Estimated Charge on Unmodified Cytochrome *c* Oxidase at Three pH Values

Residues	pK_a	pH 7.0	pH 8.3	pH 10.5
Acidic				
Asp	3.86	−61.0	−61.0	−61.0
Glu	4.25	−64.0	−64.0	−64.0
Cys	8.33	−0.2	−2.5	−5.0
Thr	10.43	0	0	−25.0
Ser	9.15	0	−6.1	−47.8
Tyr	10.07	0	0	−18.9
Amide		+62.0	+62.0	+62.0
Basic				
Arg	12.48	+31.0	+31.0	+31.0
His	6.00	+1.9	0	0
Lys	10.53	+39.0	+39.0	+19.5
Net charge		+8.7	−1.6	−109.2

acid amides could not be determined after acid hydrolysis. However, the ammonia content was a measure of acid amides, if the samples were carefully prepared for analysis. Dialysis of the sample prior to hydrolysis, the use of ammonia-free reagents, and the determination of a baseline for ammonia that the buffer might have contained, yielded an average value for ammonia of 62.0 ± 2.3 residues. The net charge at pH 7.0 calculates to be +8.7.

When the amino acid side chains are classified according to the hydrophilicity (Lys, Arg, His, Asp, Glu, Ser, Thr, Cys, Tyr) and hydrophobicity (Gly, Ala, Pro, Val, Ile, Leu, Phe, Trp, Met), the number of hydrophobic residues is 346 out of a total of 736, or 47%.

Succinylation of cytochrome oxidase was accomplished with a 500 molar excess (based on a protein molecular weight of 100,000) of succinic anhydride (Fisher Scientific Company, Springfield, New Jersey). The anhydride was ground to a fine powder in a mortar and a weighed amount was added in small portions to the oxidase solution with constant stirring. The pH was maintained at between 8.0 and 8.3 with 0.5 N NaOH. The addition took about $\frac{1}{2}$ hr and the last adjustment of pH, if needed, was 1 hr after the last addition of succinic anhydride. The solution was placed in the cold room at 4°C and was stirred constantly for 12 hr.

The succinic acid which resulted from the reaction of succinic anhydride with water as well as the surface-active agent which the enzyme preparation contained was removed either by dialysis or chromatography. Dialysis was for 4 to 5 days at 4°C with four to five changes of 0.1 M phosphate buffer, pH 8.3, at a volume ratio of one part oxidase solution to more than 30 volumes of buffer. Chromatography was either on the chloride form of Amberlite IRA-410 (Rohm and Haas, Philadelphia, Pennsylvania) or on Sephadex G-50 or G-75 (Pharmacia, Piscataway, New Jersey). The resins were equilibrated with 0.1 M phosphate buffer pH 8.3. The flow rate was adjusted to between 0.5 and 0.75 ml/min.

The percent modification by succinylation of six preparations was determined by the *O*-methylisourea method. The values ranged from 47.3 to 89.0% with an average of 67.2%. The variation is larger than might be expected for such a seemingly simple and direct reaction as succinylation.

It was not expected that succinic anhydride would alter the amino acid composition of the protein, but because of the long dialysis or of the chromatography on Sephadex G-75, it was decided to analyze preparations 10, 11, and 13. The average values are presented in Table IV. The only value that appears to deviate from the analysis for the unmodified protein is that for glutamic acid. There is no ready explanation for this exception.

The spectrum of succinylated cytochrome *c* oxidase was different from that of the unmodified enzyme. In the oxidized state the γ peak was at

TABLE IV. Amino Acid Composition of Several Succinylated Preparations of Cytochrome *c* Oxidase

Amino acid	Succinylated[a]	Unmodified[b]
Asp	60	61
Thr	52	50
Ser	51	49
Glu	59	64
Pro	34	36
Gly	53	53
Ala	58	56
Val	43	44
Met	25	24
Ile	37	37
Leu	75	73
Tyr	27	26
Phe	41	38
His	23	21
Lys	38	39
Arg	30	31

[a]Average of three preparations: 10, 11 and 13.
[b]Average of nine preparations: those presented in table II.

420 nm and in the reduced form the maxima were at 438 and 591 nm. The spectral changes occurred in two stages: (1) about 12 hr after modification, and before removal of the deoxycholate the α peak of the reduced compound was at 600 nm and the (γ peak)/(α peak) ratio of the reduced form was 6.6 (4.75 in the unmodified enzyme). All preparations were active toward ferrocytochrome *c* as a substrate in the presence of oxygen, (2) Following removal of the deoxycholate the spectrum shifted to the values given previously and the preparations had no activity. Succinylated cytochrome *c* oxidase also could not be reduced with ferrocytochrome *c* under anaerobic conditions. However, it was completely soluble in the absence of deoxycholate. The method for determining deoxycholate in this instance was made more sensitive by concentrating the oxidase solution threefold against 20% Carbowax (w/v) and by omitting the 1:4 dilution which the method calls for.

Polyacrylamide gel electrophoresis of the succinylated enzyme having no deoxycholate gave one major band which moved just behind the tracking dye. Some of the protein did not enter the gel and some was distributed throughout the upper part of the gel, indicating some denaturation and some heterogeneity.

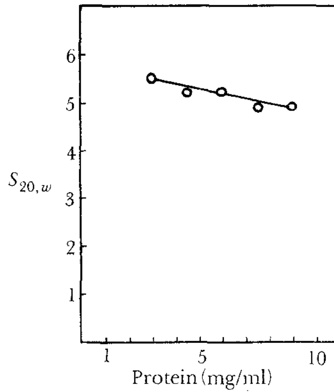

Figure 1. Concentration dependence of the sedimentation coefficient (preparation 12).

Sedimentation of the succinylated enzyme in the analytical ultracentrifuge was slower than that of the unmodified preparations which had an average $S_{20,w}$ value of 8.4. A few of the unmodified preparations had a slow moving component with an $S_{20,w}$ of 4.8. The $S_{20,w}$ and $D_{20,w}$ values for the succinylated enzyme were concentration dependent, as can be seen in Figs. 1 and 2. Calculated molecular weights for three preparations are presented in Table V. The average value of 126,150 indicates that the succinylated protein was present as the monomer. There was some heterogeneity in both the succinylated and unmodified enzymes, as evidenced by excessive spreading of the sedimentation patterns. Preliminary succinylated preparations showed skewing of both the sedimentation and diffusion patterns and therefore all preparations were precentrifuged in the Beckman Model L preparative ultracentrifuge to remove heterogeneous material.

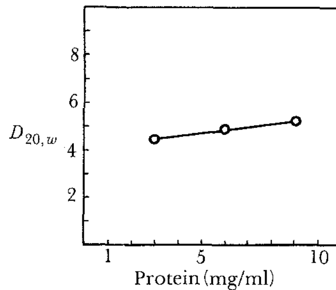

Figure 2. Concentration dependence of the diffusion constant (preparation 12).

TABLE V. Molecular Weights of Several Preparations of Succinylated Cytochrome *c* Oxidase

Preparation	$S^0_{20,w}$	$D^0_{20,w}$	Molecular weight
10	5.5	(5.3)[a]	–
12	5.8	4.1	123,073
13	5.8	3.9	129,236
14	6.0	4.0	130,350
3	5.8	[b]	–
T–1	6.0	[b]	–
Average	5.8 ± 0.1	4.0 ± 0.07	126,150 ± 4,384

[a] Not extrapolated to zero concentration of protein.
[b] Omitted because of skewing of peak.

Discussion

The fractionation with ammonium sulfate of cytochrome *c* oxidase which has been solubilized with sodium deoxycholate may lead to preparations of low activity, probably because the amount of deoxycholate attached to the pellet is variable and still large. Since a deoxycholate determination which can be used to adjust the deoxycholate content would take too long, i.e., the enzyme would deteriorate still further, the recommended procedure is to chromatograph immediately on Sephadex G-75 equilibrated overnight with the desired amount of deoxycholate (usually 0.5%). The oxidase may also be transferred into Tween 80 at this stage, if desired, by the use of a column. If for any reason chromatography cannot be done immediately, the effects of deoxycholate may at least partially be overcome by dissolving the final pellet from the ammonium sulfate fractionation in buffer containing 0.5% Tween 80.

The exact molecular weight of cytochrome *c* oxidase is not known. Values ranging from 200,000 [9] to 321,000 [8] have been reported. It is not surprising then that the minimum molecular weight based on the heme content varies as much, if not more. The many values reported in the literature are presented in Table VI. It is with this collection of values that we wish to compare the minimum molecular weights obtained in the present study by amino acid analysis, and by ultracentrifugation after succinylation.

The amino acid analyses of the unmodified preparations were corrected for time-dependent losses due to hydrolysis. The average variations of the analyses were usually no more than three residues, except for aspartic acid, glutamic acid, threonine and serine, which are known to be particularly

TABLE VI. Minimum Molecular Weight of Cytochrome *c* Oxidase Based on Heme Content

Molecular weight (daltons)	Investigators	Reference
108,700–122,700	Griffiths and Wharton (1961)	22
138,400	Yonetani (1961)	23
113,800	Fowler et al. (1962)	24
69,300–128,600	Wainio (1964)	25
92,000	Matsubara et al. (1965)	10
111,100–125,000	Sun et al. (1968)	26
119,000	Love et al. (1970)	9
125,000	Zamudio and Williams (1971)	28
124,000	Kierns et al. (1971)	27
110,000	Wainio et al. (1973)	11
68,500–75,800	Komai and Capaldi (1973)	29
73,500	Ozawa et al. (1975)	30

labile during acid hydrolysis. The machine error was ±3% and most of the variations fall within this range.

The average protein molecular weight per heme as calculated from the amino acid composition of nine preparations is 83,967. When the heme, ammonia, and cysteine are added the minimum molecular weight is increased to 86,477. This value compares favorably with that obtained by Matsubara et al. [10] by amino acid analysis and is only somewhat lower than the average value for all preparations listed in Table VI. Love et al. [9] reported a molecular weight of 100,000 by the sedimentation method for the monomer of cytochrome *c* oxidase which they prepared by exposing the enzyme in Emasol (or Tween) to pH 9.5–11.

The basic character of cytochrome *c* oxidase, which has previously been pointed out by Matsubara et al. [10] is evident from the fact that the calculated isoelectric point is at about pH 8.1.

The high content of hydrophobic residues (47%) may explain in whole or in part why cytochrome *c* oxidase is an insoluble membrane-bound protein. Capaldi and Vanderkooi [31] pointed out that in their calculation of polarities for 224 proteins, cytochrome *c* oxidase was one of several membrane proteins with a low polarity. In contrast, only 2% of the 205 soluble proteins for which they had data had as low a polarity. They based their calculation for cytochrome *c* oxidase on the data of Matsubara et al. [10].

Succinylated cytochrome *c* oxidase is completely soluble in the absence of deoxycholate. The protein may be freeze-dried, stored at −15°C, and redissolved in water to yield a clear solution. Its molecular weight as

determined from sedimentation and diffusion data is 126,000. When compared with the molecular weight of unmodified cytochrome *c* oxidase, i.e., 228,000 daltons, as determined in our laboratory [11], and with the minimum molecular weights presented in Table VI, it is apparent that succinylated oxidase has the size of the monomer, provided cytochrome *c* oxidase has two equal subunits.

The degree of modification by succinylation was found not to be proportional to the amount of succinic anhydride added, as determined in preliminary experiments not reported herein. Even with a constant 500 molar excess of anhydride, the degree of modification of lysyl residues varied from 47.3 to 83.5%. The reason for this variability is not known.

The existence of the succinylated preparation predominantly as the monomer may be related in part at least to the charge on the protein. Love et al. [9] produced a monomer by raising the pH of their Emasol-solubilized cytochrome *c* oxidase to between 9.5 and 11. As presented in Table III the unmodified enzyme has a net charge of −109.2 at pH 10.5. A similar calculation for the succinylated preparation with 50% of the lysyl residues modified yields a net charge of −40.6 at pH 8.3.

Succinylated cytochrome *c* oxidase from which the deoxycholate has been subsequently removed is virtually inactive toward ferrocytochrome *c* as a substrate. However, succinylation per se does not destroy the activity provided the deoxycholate is not removed. The greatest retention of activity was 75% in a preparation not reported herein which had 51% of its lysine residues modified. The activity was present 24 hr after the initial addition of the anhydride when the reaction mixture was kept in the cold room at 4°C. Following removal of the succinic acid and the deoxycholate by chromatography, the activity decreased to between 1 and 2% of the control value. Reactivation with either deoxycholate or Tween was not tested.

Acknowledgments

Supported by grants from the United States Public Health Service (AM 9762), the Rutgers University Research Council, and the Charles and Johanna Busch Memorial Fund to the Bureau of Biological Research.

References

1. I. M. Klotz, *Methods Enzymol.,* **11** (1967) 576.
2. A. Gounaris and M. Ottesen, *C. R. Trav. Lab. Carlsberg,* 35 (1965) 37.
3. I. M. Klotz and S. Keresztes-Nagy, *Biochemistry,* 2 (1964) 445.
4. A. F. Habeeb, H. G. Cassidy, and S. J. Singer, *Biochim. Biophys. Acta,* 29 (1958) 587.

5. D. H. MacLennan, A. Tzagoloff, and J. Rieske, *Arch. Biochem. Biophys.*, **109** (1965) 383.
6. D. H. MacLennan and A. Tzagoloff, *Biochim. Biophys. Acta,* **96** (1965) 166.
7. Y. Kagawa, *Biochim. Biophys. Acta,* **131** (1967) 586.
8. A. Tzagoloff, P. C. Yang, D. C. Wharton, and J. S. Rieske, *Biochim. Biophys. Acta,* **96** (1965) 1.
9. B. Love, S. Chan, and E. Stotz, *J. Biol. Chem.*, **245** (1970) 6664.
10. H. Matsubara, Y. Orii, and K. Okunuki, *Biochim. Biophys. Acta,* **97** (1965) 61.
11. W. W. Wainio, T. Laskowska-Klita, J. Rosman, and D. Grebner, *J. Bioenergetics,* **4** (1973) 455.
12. W. W. Wainio, S. J. Cooperstein, S. Kollen, and B. Eichel, *J. Biol. Chem.*, **173** (1948) 145.
13. D. Keilin and E. F. Hartree, *Proc. Roy. Soc., Ser. B,* **125** (1938) 171.
14. C. R. Szalkowski and W. J. Mader, *Anal. Chem.*, **235** (1952) 1602.
15. L. T. Kremzner and W. W. Wainio, *Biochim. Biophys Acta,* **52** (1961) 208.
16. J. Opieńska-Blauth, M. Chareziński, and H. Barbeć, *Anal. Biochem.*, **6** (1963) 69.
17. J. Spies, *Anal. Chem.*, **39** (1967) 1412.
18. B. J. Davis, *Ann. N.Y. Acad. Sci.*, **121** (1964) 404.
19. H. R. Maurer, in *Disc Electrophoresis,* K. Fishbeck, ed., Walter de Gruyter, Berlin (1971).
20. S. Takemori, I. Sekuzu, and K. Okunuki, *Biochim. Biophys. Acta,* **51** (1961) 464.
21. W. W. Wainio, B. Eichel, and A. Gould, *J. Biol. Chem.*, **235** (1960) 1521.
22. D. E. Griffiths and D. C. Wharton, *J. Biol. Chem.*, **236** (1961) 1857.
23. T. Yonetani, *J. Biol. Chem.*, **236** (1961) 1680.
24. L. R. Fowler, S. H. Richardson, and Y. Hatefi, *Biochim. Biophys. Acta,* **64** (1962) 170.
25. W. W. Wainio, *J. Biol. Chem.*, **239** (1964) 1402.
26. F. F. Sun, K. S. Prezbindowski, F. L. Crane, and E. E. Jacobs, *Biochim. Biophys. Acta,* **153** (1968) 804.
27. J. J. Kierns, C. S. Yang, and M. V. Gilmour, *Biochem. Biophys. Res. Commun.*, **45** (1971) 835.
28. I. Zamudio and G. R. Williams, *Arch. Biochem. Biophys.*, **143** (1971) 240.
29. H. Komai and R. A. Capaldi, *FEBS Lett.*, **30** (1973) 273.
30. T. Ozawa, M. Okumura, and K. Yagi, *Biochem. Biophys. Res. Commun.*, **65** (1975) 1102.
31. R. A. Capaldi and G. Vanderkooi, *Proc. Natl. Acad. Sci. U.S.A.*, **69** (1972) 930.