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Deaggregation of Mammalian Cytochrome *c* Heme Undecapeptide*

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Abstract

Aggregation of the oxidized heme undecapeptide of mammalian cytochrome *c* in aqueous solution has been demonstrated by other investigators. Our results indicate that it is the α-amino group on the terminal valine which participates in the aggregation reaction. Fully deaminated (valine and lysine) heme undecapeptide is deaggregated, i.e., it is in the monomeric state, while partially valine-deaminated heme undecapeptide has the circular dichroic spectrum of a mixture of the deaggregated and aggregated species.

Introduction

The aggregation of the oxidized heme undecapeptide§ of cytochrome *c* has been shown to be a function of concentration [1], pH [2], and temperature [3]. The wavelength of the maximum of the γ-peak in the spectrum of the oxidized compound is assumed to reflect the state of

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§ Heme undecapeptide prepared with pepsin (H11P):
Val–Gln–Lys–Cys–Ala–Gln–Cys–His–Thr–Val–Glu
(the two Cys residues linked by —Heme—)

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aggregation. Harbury and Loach [1] obtained a final position of 398 nm on dilution at pH 6.9 and 395 nm at pH 3.0. They, as well as Ehrenberg and Theorell [2], and Paleus et al. [3], concluded that the species obtained on dilution was the oxidized monomer.

Ehrenberg and Theorell [2] studied the sedimentation of the oxidized heme undecapeptide at pH values of 2.3–8.7. The $S_{20,w}$ value at pH 2.3 was 0.60 from which a molecular weight of approximately 2000 daltons was calculated. At pH 8.7 the $S_{20,w}$ was equal to 1.57 and a molecular weight near to that of a pentamer was obtained. Margoliash et al. [4], however, obtained sedimentation coefficients at several pH values which were approximately the same and argued against the idea that the spectral changes were due to aggregation.

Urry [5] studied the undecapeptide by ORD and CD spectrometry and found that the oxidized form showed a three-fold splitting in the region of the γ-peak in the polymeric state, and no splitting in the monomeric state. Urry concluded that the distance between heme centers of the polymer was 16 Å, and the arrangement was head to tail (heme to amino-acid residue) rather than the more characteristic stacking (heme to heme) found in most heme compounds. Urry suggested that the ϵ-amino group of lysine supplied the ligand for polymerization. Our results suggest that it is the α-amino group on the terminal valine which is responsible for the aggregation.

Methods and Materials

Equine-heart cytochrome *c* was Type VI purchased from the Sigma Chemical Co., St. Louis. The unmodified heme undecapeptide was prepared with pepsin according to Harbury and Loach [6], and the amino-acid analysis (Table I) is consistent with the published values. Deamination to produce the one modified heme undecapeptide|| was accomplished by treatment with nitrous acid as described by Anfinsen et al. [7]. Transamination was used to remove the terminal-amino group on the valine by the method of Dixon [8], who recommended the use of 0.1 M glyoxylic acid, 2 mM $CuSO_4$, 2 M sodium acetate and 0.4 M acetic acid at pH 5.5. The reaction proceeded for 30 min at room temperature. Visible spectra were recorded with a Cary model 11 spectrophotometer (1 cm cells) and CD spectra with a Cary model 60 spectropolarimeter (1 mm or 5 cm cells). Ultracentrifugal analyses were made with a Beckman model E analytical ultracentrifuge using an epon-filled double-sector synthetic boundary cell. Amino acid analyses were made with a Beckman model 120B analyzer.

Valine- and lysine-deaminated heme undecapeptide (VLDH11P); and partially valine-deaminated heme undecapeptide (½VDH11P).

TABLE I. Amino acid analyses of the unmodified and deaminated heme undecapeptides of cytochrome *c*

Amino acid	H11P	VLDH11P	½VDH11P
Lysine	0.97[a]	0.09	1.00
Histidine	0.94	1.00	0.97
Aspartic acid	0.06	0.06	0.05
Threonine	0.93	0.85	0.89
Serine	0.09	0.14	0.13
Glutamic acid	2.98	2.83	3.01
Glycine	0.06	0.20	0.15
Alanine	1.00	1.00	1.00
Cysteine	1.47	0.62	1.17
Valine	2.14	1.00	1.64

[a] Based on alanine taken as 1.00.

Results

Table I presents the results obtained following HNO_2 treatment (VLDH11P). The ϵ-amino group of lysine is almost completely removed, as is the N-terminal amino group of one valine, while histidine is relatively unchanged. No separate determinations were made of the two glutamines. However, Flatmark [9] reported that HNO_2 treatment results in deamination.

Table I also records the result of reacting H11P with glyoxylic acid in the presence of Cu^{2+} at pH 5.5. One-half residue of valine was lost (½VCH11P). Attempts to increase the extent of deamination of the valine were unsuccessful. However, it should be noted that the method is highly selective in that no lysine was deaminated.

The change in spectral characteristics produced by deamination are given for the oxidized and reduced heme undecapeptide at pH 7.5 in Fig. 1. The γ-peak of the oxidized form is shifted from 402 nm to a shorter wavelength and this shift is similar to that obtained on dilution or acidification. The most striking change, however, takes place in the spectrum of the reduced form where the γ-peak appears to be split into two peaks, and the α- and β-peaks at 550 nm and 520 nm, respectively, are poorly developed. In contrast, the partially valine-deaminated heme undecapeptide had spectra very much like those of the unmodified undecapeptide.

The effect on the γ-peak of a 1000-fold dilution at pH 7.5 is presented in Table II for the HNO_2-modified, the glyoxalate-reacted, and the unmodified heme undecapeptide. No shift of the γ-peak maximum is observed with the fully deaminated species while the concentration dependence of the unmodified form is quite apparent. The fully deaminated heme undecapeptide has a constant maximum at 397 nm

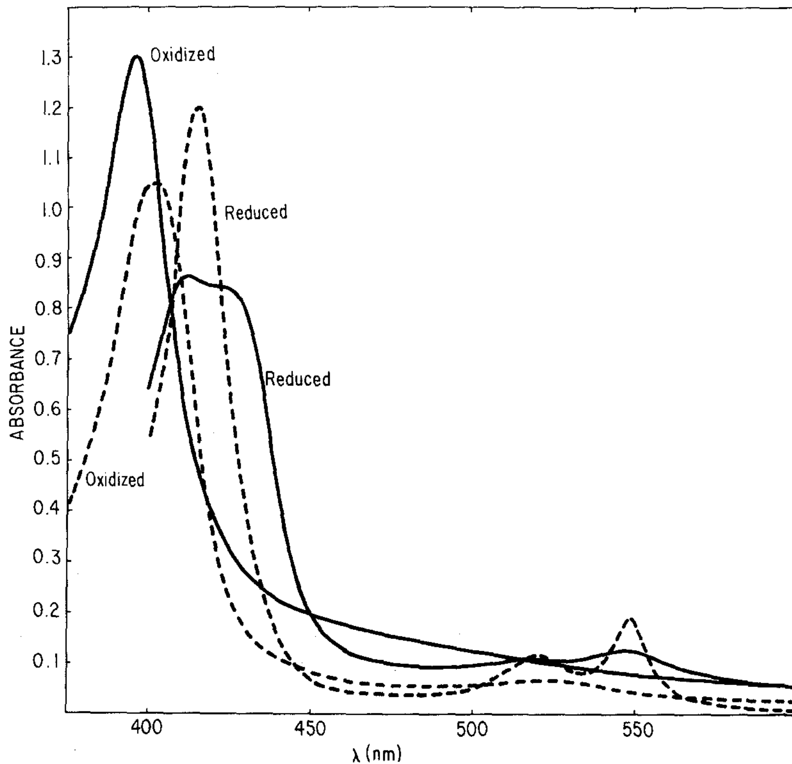

Figure 1. Spectra of oxidized and reduced heme undecapeptide (– –) of equine-heart cytochrome *c* at a concentration of $\sim 1 \times 10^{-5}$ M in 0.196 M phosphate, pH 7.5. Spectra of oxidized and reduced deaminated heme undecapeptide (——) of equine-heart cytochrome *c* at a concentration of $\sim 5 \times 10^{-5}$ M in 0.196 M phosphate, pH 7.5.

which is one nm less than the value given by Harbury and Loach as being characteristic of the monomeric form. In contrast, the partially valine-deaminated heme undecapeptide had an asymmetric γ-peak at the high concentrations indicative of there being two species present, the 407 nm aggregated species and the 397 nm deaggregated species.

Ultracentrifugation studies were carried out on the oxidized forms of the HNO_2-treated and the unmodified heme undecapeptide. The $S_{20,w}$ value obtained for the deaminated oxidized form, 0.35, and for the unmodified form, 1.84, at pH 7.5 in 0.196 M phosphate support the expectation that the fully deaminated form is monomeric and the untreated form is polymeric.

TABLE II. Effect of dilution at pH 7.5 on the position of the γ-peak in the absorption spectrum of the oxidized unmodified (H11P), oxidized fully deaminated (VLDH11P), and oxidized partially valine-deaminated (½VDH11P) heme undecapeptides of cytochrome *c*

H11P		VLDH11P		½VDH11P	
Concentration (M)	γ-Peak (nm)	Concentration (M)	γ-Peak (nm)	Concentration (M)	γ-Peak (nm)
2.5×10^{-4}	407	5×10^{-4}	397	1.25×10^{-4}	404[a]
2.5×10^{-5}	405	5×10^{-5}	397	1.25×10^{-5}	400[b]
2.5×10^{-6}	398	5×10^{-6}	397	1.25×10^{-6}	397[c]
2.5×10^{-7}	397	5×10^{-7}	397	1.25×10^{-7}	397[c]

[a] Asymmetric; skewed toward lower wavelength.
[b] Asymmetric; skewed toward higher wavelength.
[c] Symmetric.

Since Urry [5] used CD measurements to study the possible aggregation of the unmodified heme undecapeptide, similar measurements were made here to show that the fully deaminated form could not aggregate. In the unmodified form the γ-peak gives the complexity shown in Fig. 2A, curve 2, while the deaminated form gives a simple peak (Fig. 2B, curve 2). Thus our results never show a complexity for the wholly deaminated heme undecapeptide (Fig. 2B) under conditions where the unmodified counterpart gives a well-defined splitting in the region of the γ-peak, i.e., at concentrations of 2.5×10^{-4} and pH values of 8.88 and 7.5. At pH 7.5 (Fig. 2A, curve 1) the unmodified heme undecapeptide shows a different type of splitting in the γ-peak region than at pH 8.8 (Fig. 2A, curve 2). Dilution at pH 7.5 does not result in conversion to the simpler, monomeric form (Fig. 2A, curve 4), as is the case at the higher pH (Fig. 2A, curve 3). In Fig. 2C the corresponding curves for the partially valine-deaminated heme undecapeptide are presented. The curves are much less complex than those in Fig. 2A, but not as simple as those in Fig. 2B. Curve 1 in Fig. 2C could be constructed by assuming it is a mixture of the aggregated (Fig. 2A, curve 1) and the deaggregated (Fig. 2B, curve 2) forms. Curve 2 in Fig. 2C shows that there is present much less of the complex aggregated form than there is in a solution of the unmodified heme undecapeptide at the same concentration (Fig. 2A, curve 2).

The effects of a number of ligands on the spectrum of the fully deaminated heme undecapeptide are presented in Table III. Surprisingly, the addition of L-histidine methyl ester resulted in a spectrum more like that of cytochrome *c* than any of the other ligands, including *N*-acetyl methionine methyl ester and DL-methionine. Notably, L-histidine methyl ester produced a Soret peak for the oxidized form at 404.5 nm, a

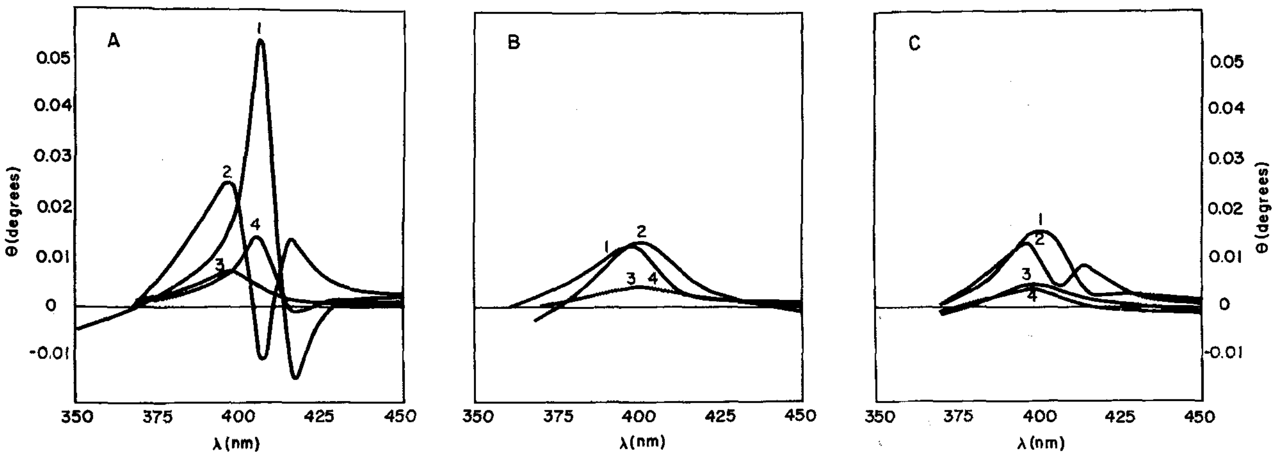

Figure 2. Circular dichroic spectra of unmodified heme undecapeptide (A), wholly deaminated heme undecapeptide (B), and partially valine-deaminated heme undecapeptide (C). Curves 1, phosphate buffer pH 7.5, $\sim 2.5 \times 10^{-4}$ M undecapeptide, 1 mm cell; curves 2, bicarbonate buffer pH 8.9, $\sim 2.5 \times 10^{-4}$ M undecapeptide, 1 mm cell; curves 3, bicarbonate buffer pH 8.9, $\sim 1.25 \times 10^{-6}$ M undecapeptide, 5 cm cell; curves 4, phosphate buffer pH 7.5, $\sim 1.25 \times 10^{-6}$ M undecapeptide, 5 cm cell. Temperature 10° C.

TABLE III. Effect of ligands at pH 7.5 on the absorption maxima of the oxidized fully deaminated heme undecapeptide (VLDH11P) of cytochrome *c*

Compound	Oxidized (nm)	Reduced γ-Peak (nm)	Reduced β-Peak (nm)	Reduced α-Peak (nm)	Oxidized γ:reduced γ
VLDH11P	397	412 & 424 twin	520	548	1.36
VLDH11P + N-acetyl methionine methyl ester[a]	399	415 with 424 shoulder	522	551	1.00
VLDH11P + *L*-methionine[a]	398	415 with 424 shoulder	522	550	1.20
VLDH11P + glycine[a]	398	414 & 424 twin	521	549	1.39
VLDH11P + *L*-histidine methyl ester[a]	405	414 single	520	548	0.91
Cytochrome *c*	408	415	520	550	0.82

[a] >500 molar excess.

Soret peak for the reduced form at 414 nm, and a ratio of the height of the oxidized peak to the reduced peak of 0.91. These values are 408 nm, 415 nm, and 0.82, respectively, for cytochrome *c*.

Discussion

Complete deamination of heme undecapetide produces a species which appears to be monomeric at concentrations and pH values at which the unmodified form is aggregated. The criteria used to determine the monomeric state were: (a) lack of change in the position of the γ-peak maximum following a 1000-fold dilution; (b) $S_{20,w}$ values which are consistent with the values Ehrenberg and Theorell [2] ascribed to the monomer; (c) lack of splitting of the CD spectrum in the γ-peak region—splitting is believed to be characteristic of an aggregating system [5].

The unmodified heme undecapeptide is assumed to aggregate because only five of the six coordination positions around the heme of the monomer are filled. The open position can be filled by a group which has a free electron pair. Two amino groups, the α- and ε-amino groups of the valyl and lysyl residues, respectively, are present on the heme undecapeptide.

Two types of amino group-heme interactions are considered possible—intermolecular and intramolecular [3]. The first type must be

ruled out on the basis of our data for the oxidized form, even though Margoliash et al. [4] have presented results which can be used to support an intermolecular model. The second type requires two molecules, one which supplies the unprotonated amino group, and the other, the open 6th coordinating position. A polymer of n units can be envisioned where n may be determined by solubility.

Our results do indicate that an amino group is required for polymerization. Since, coordination requires an unprotonated amino group, pH considerations should enable one to select the proper group. Paleus et al. [3] titrated heme undecapeptide and reported the following pK values: 9.6 for the lysyl ϵ-amino and 7.6 for the valyl α-amino group. If the 100% monomeric species has a maximum at 395 nm at pH 2.3 and the 100% pentameric or hexameric species [2] a maximum at 408 nm at pH 9.8, and if the equilibrium at any pH is mainly between monomer and pentamer or hexamer, then the Henderson-Hasselbach relationship can be used to calculate the position of the absorption maximum at a given pH. The polymer concentration is taken to be the concentration of base in the Henderson-Hasselbach equation, since, even though it consists of five or six peptide units, at least one of the subunits must contain an unprotonated amino group. When the α-amino group is assumed to be the instrument of polymerization, the predicted γ-peak maximum at pH 7.5 would be at 402.5 nm. Paleus et al. [3] reported a γ-peak maximum at pH 7.5 of 402 nm at a concentration of 2.5×10^{-6} M and we obtained a γ-peak maximum of 405 nm at pH 7.5 and 2.56×10^{-5} M. By making the same assumption for the ϵ-amino group, the absorption maximum would be at 395.1 nm. Even our most dilute sample (2.56×10^{-7} M) gives a γ-peak at 397 nm. Harbury and Loach [1] reported a final value of 398 nm at pH 6.9.

The results with partially valine-deaminated heme undecapeptide strongly support the suggestion that it is the terminal α-amino group which is responsible for aggregation. Deamination of approximately one-half of the N-terminal valine residues gives a product which has CD curves much less complex than those for the aggregated unmodified heme undecapeptide. Furthermore, the shifts in the maximum of the visible absorption spectrum curve following dilution were much less pronounced with the partially valine-deaminated heme undecapeptide than with the unmodified heme undecapeptide. In fact, the visible absorption spectrum curves at the higher concentrations of the partially valine-deaminated heme undecapeptide could be interpreted as being the sum of a deaggregated (397 nm) and an aggregated species (407 nm).

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