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The Stoichiometry of Binding of the Aurovertins to Mitochondrial ATPases: Revision of the Molar Absorption Coefficient

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Abstract

The purity of aurovertins A, B, and D, which inhibit the mitochondrial ATPase and show fluorescence enhancement when bound, has been determined by high-resolution proton nuclear magnetic resonance. This technique demonstrated that solvent molecules, especially water, are tightly bound to crystalline aurovertins B and D. The molar absorption coefficient for aurovertins A, B, and D at the longest wavelength maximum (367.5–369 nm) has been determined to be 38,500 liter · mol^{-1} · cm^{-1}. This correct molar absorption coefficient should enable correct binding stoichiometries for aurovertin to ATPases to be determined.

Key Words: ATPase; aurovertin; binding stoichiometry; molar absorption coefficient; nuclear magnetic resonance (NMR)

Introduction

Crude aurovertin as isolated by Baldwin *et al.* (1964) has been shown to be a mixture, the major components of which have been designated aurovertins A, B, C, D, and E (Osselton *et al.*, 1974; Linnett and Beechey, 1979).

The aurovertin used for earlier studies (kindly distributed by Dr. H. A. Lardy) has been shown to be mainly aurovertin D (Osselton *et al.*, 1974). Aurovertin B, which has been produced predominantly in our hands from *Calcarisporium arbuscula* (Osselton *et al.*, 1974), has almost identical biological properties to aurovertin D (Linnett *et al.*, 1977; Linnett and Beechey, 1979).

The usefulness of aurovertins B and D as specific and potent inhibitors of the mitochondrial ATPase has been increased by the large enhancement of

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Table I. Molar Absorption Coefficients Reported in the Literature for Aurovertins

Aurovertin	mp (°C)	Absorption data as presented[a]				Molecular weight[b] used	Reference
		$\epsilon(1)$	$\lambda(1)$	$\epsilon(2)$	$\lambda(2)$		
B	166.5–168.5	34,400	372	28,200	270	460.5	Mulheirn *et al.* (1974)
B	166.5–168.5	34,850	372	31,500	270	460.5	Mulheirn (unpublished results)
D	195–197	42,700	367.5	34,200	270	490.5	Baldwin *et al.* (1964)
D	195–205	36,000	367.5	32,800	272.5	490.5	Bertina (1972), ex H. A. Lardy
D	120–130	29,000	36.75	26,000	272.5	490.5	Bertina (1972)
D	—	28,500	367.5	—	—	490.5	Muller *et al.* (1977)
D	—	33,200	368	—	—	—	Douglas *et al.* (1977)
D	—	35,100	368	30,000	270,275	476.5	Sartre *et al.* (1980)

[a]Absorption data were obtained from solutions in absolute ethanol. Units: ϵ values in liter $\cdot$ mol^{-1} $\cdot$ cm^{-1}, λ values in nm.
[b]Correct molecular weight for aurovertin D = 476.5 (Beechey *et al.*, 1967)

fluorescence observed when they are bound to the mitochondrial ATPase (Lardy and Lin, 1969). Fluorescence enhancement has enabled studies to be made on the stoichiometry of binding of the aurovertins to the ATPase (see, for instance, Chang and Penefsky, 1973) and to the isolated β-subunit (Verschoor *et al.*, 1977; Douglas *et al.*, 1977).

In these studies measurements of the concentration of the aurovertin solutions have relied on the value of the molar absorption coefficient (ϵ) of aurovertin D for the long-wavelength, broad absorption maximum at about 369 mm. Despite the publication of the precise molecular weight of aurovertin D (Beechey *et al.*, 1967) followed by its structure (Mulheirn *et al.*, 1974), it can be seen from Table I that the published ϵ values for aurovertins B and D vary from 27,700 to 41,000 liter $\cdot$ mol^{-1} $\cdot$ cm^{-1}; the largest value is 50% greater than the smallest. It is obvious that such a spread of ϵ values is unacceptable for stoichiometry measurements. Since the molar absorption coefficient for a given compound in a specified solvent at a certain wavelength is an absolute parameter, it was decided to determine the correct ϵ value for the aurovertins.

Experimental

Purification of Aurovertins

Aurovertins B and D were obtained from *Calcarisporium arbuscula* and separated initially by preparative TLC[2] (Linnett and Beechey, 1979). Aurovertin B was recrystallized several times from acetone and finally from methanol. Aurovertin D was further purified by semipreparative HPLC on a 25 $\times$ 0.95 cm stainless steel column packed with 10μm-diameter silica gel (Partisil 10; Whatman, Maidstone, Kent, U.K.). Approximately 8 mg of crude aurovertin D dissolved in 500 μl of chloroform was injected each time. The eluting solvent was chloroform/acetone (7:3, v/v) at a flow rate of 6.0 ml/min. The peak corresponding to aurovertin D was detected spectrophotometrically at 420 nm and collected. The purified aurovertin D was recrystallized from diethyl ether with some difficulty.

Aurovertin A, which occurs naturally in the aurovertin mixture (Osselton *et al.*, 1974), was synthesized by acetylation of purified aurovertin B with acetic anhydride in pyridine. After purification by preparative TLC on silica gel with ethyl acetate/toluene (7:3, v/v), aurovertin A was recrystallized from absolute ethanol.

[2] Abbreviations: HPLC, high-performance liquid chromatography; mol. wt., molecular weight; NMR, nuclear magnetic resonance; ppm, parts per million; TLC, thin layer chromatography.

Purity of Aurovertins

The samples of aurovertins B and D were at least 95% pure by HPLC on the Partisil column described above using the same solvent and flow rate with detection at 369 nm. Their purity was also checked by analytical HPLC on a 10 × 0.45 cm stainless steel column packed with 5μm-diameter Lichrosorb RP-8 (BDH Chemicals Ltd., Poole, Dorset, U.K.) and eluted with a linear gradient of 37.7:62.3 (v/v) acetonitrile/H_2O to 46.5:53.5 (v/v) acetonitrile/H_2O in 10 min at a constant flow rate of 1.7 ml/min. Aurovertin B showed a single peak eluting at 5.7 min, with detection at 369 nm. Aurovertin D showed a single peak eluting at 2.2 min, with detection at 369, 272, and 216 nm.

Measurements of UV Absorption Spectra

Samples of crystalline aurovertins (approximately 200 μg) dried as described in the Results section were weighed in clean aluminum boats using an electronic autobalance AD-2Z (Perkin-Elmer Ltd., Beaconsfield, Bucks, U.K.) to an accuracy of ±1 μg (±0.5%) and then dissolved in 25 ml of absolute ethanol (James Burrough Ltd., London, U.K.). Absorbance measurements were made in 10mm-pathlength silica cuvettes with absolute ethanol in the reference cell and bandwidth 1 nm, in the dual-beam mode using an Acta MVI spectrophotometer (Beckman-RIIC Ltd., High Wycombe, Bucks, U.K.). The instrumental absorbance readout was corrected where necessary by measuring the absorbance at that wavelength of a standard solution of K_2CrO_4 in 0.05 N KOH against water in the reference cell (Graselli, 1973).

Solvent Measurements by NMR Spectroscopy

^{1}H-NMR spectra were obtained at 360 MHz on a WH 360 spectrometer (Bruker Spectrospin Ltd., Coventry, U.K.) at 20°C in the Fourier transform mode. Initially, the spectrum of 0.5 ml of the pure solvent, [^{2}H]chloroform ("Chloroform-d, 100%," containing a minimum of 99.96 atom % ^{2}H; Merck, Sharp & Dohme Canada Ltd., Cambrian Chemicals, Croydon, U.K.), was obtained and the peaks due to residual $CHCl_3$ and H_2O were integrated. Chemical shifts were referenced to the $CHCl_3$ peak, which had been assigned to δ7.259 ppm relative to tetramethylsilane in an earlier experiment.

Determination of the Water Content of the Aurovertins

The NMR spectrum of the solvent, [^{2}H]$CHCl_3$, was accumulated (see traces A, Figs. 1 and 2). From this spectrum the ratio of the integrated proton signals from the contaminating [^{2}H]$CHCl_3$ and water in the deuterated solvent was calculated. Let this ratio be A.

An unweighed sample of an aurovertin (0.5–2 mg) was then dissolved in

the $[^2H]CHCl_3$ and its NMR spectrum was accumulated (see traces B, Figs. 1 and 2). This time, the "water signal" was made up of contributions from:

1. the water contaminating the solvent
2. the water contained in the aurovertin crystals
3. any signal in this region caused by the presence of the aurovertin.

The ratio of the integrated proton signals from the contaminating $[^2H]CHCl_3$ and the "water" was measured again. Let this ratio be B.

Now $B - A$ is the sum of the signals from the aurovertin and any water of crystallization, which has been normalized to the integral of the absorption of the $[^1H]$chloroform proton. (It is necessary to normalize the integrals from the different spectra in this way since for operational reasons the spectra were all obtained at different sensitivity settings on the NMR spectrometer.)

The average value of the integrated signals from one hydrogen atom in the aurovertin spectrum was then calculated. This value was again normalized to the $[^1H]CHCl_3$ integral. Let this ratio be C.

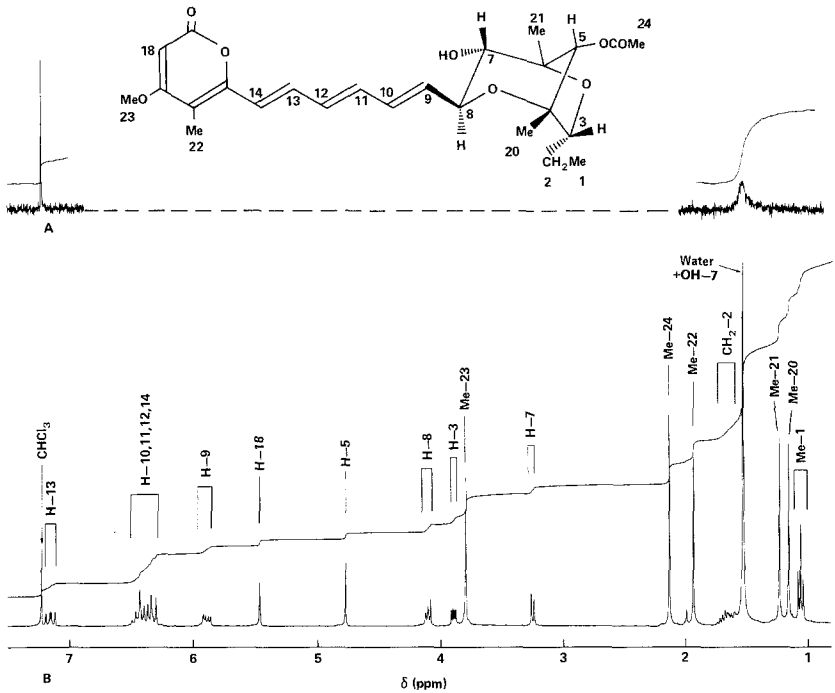

Fig. 1. ^{1}H-NMR spectrum of recrystallized aurovertin B in $[^2H]CHCl_3$. Trace A. The spectrum of the $[^2H]CHCl_3$ solvent before the addition of aurovertin. Trace B. This spectrum was accumulated for the higher concentration of a pair of sequential solutions as described in the text. Assignments are made for all the observed signals.

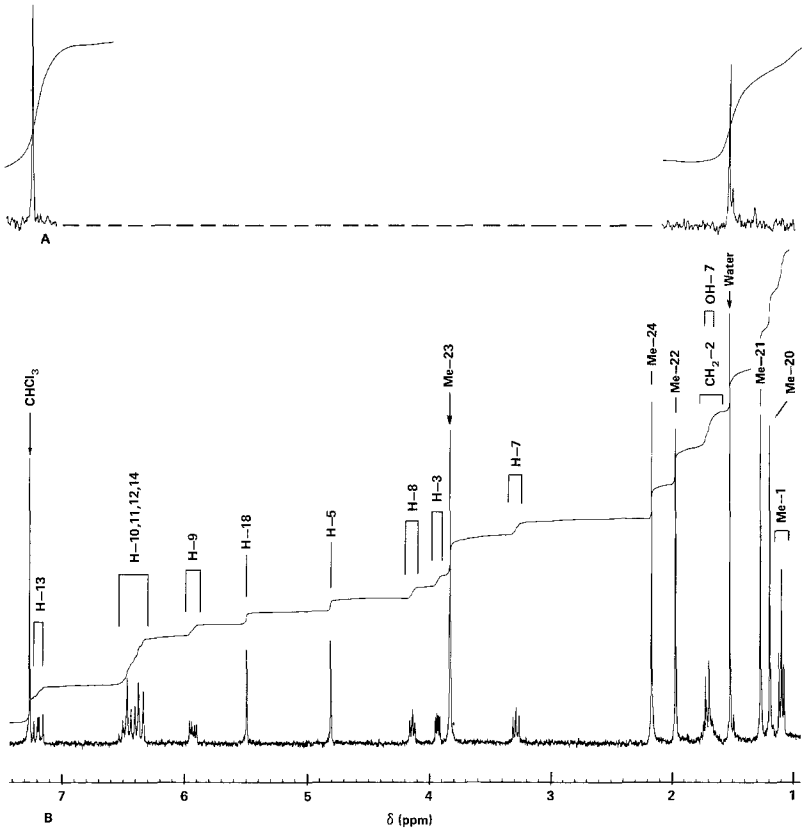

Fig. 2. ^{1}H-NMR spectrum of rigorously dried aurovertin B in [^{2}H]$CHCl_3$. Trace A. The spectrum of the [^{2}H]$CHCl_3$ solvent before the addition of aurovertin. Trace B. This spectrum was accumulated for the lower concentration of a pair of sequential solutions.

Now $(B - A)/C$ is the absolute number of hydrogen atoms which absorb at that position in the NMR spectrum.

This procedure was carried out at two concentrations of each aurovertin, simply to give added confidence to the estimation.

Results

Estimation of the Molar Absorption Coefficient of Aurovertin B

Preliminary data on the structure of aurovertin B obtained by X-ray crystallography indicated the presence of 3–4 water molecules per aurovertin molecule in material which had been recrystallized from acetone (R. Norrestam, personal communication).

A sample of aurovertin B which had been crystallized finally from methanol and shown to be pure by HPLC (see Experimental) was dried *in vacuo* (less than 0.13 Pa, 1 μm Hg) for 1 h at 20°C over silica gel. The purity of this sample was analyzed also by ^{1}H-NMR at 360 MHz in [^{2}H]$CHCl_3$. The peaks in the spectrum were assigned as shown in Fig. 1 following Mulheirn *et al.* (1974). No significant proton containing impurity other than water was detected.

Examination of the NMR spectrum of aurovertin B shown in Fig. 1 reveals that the absorption due to the 7-OH proton is combined with the absorption due to the water. Hence the values of $(B - A)/C$ were reduced by 1 to give the number of water hydrogen atoms present in the aurovertin sample.

The number of molecules of water of crystallization present in the aurovertin B samples is approximately 3 (see Table II). Hence the molar absorption coefficients of aurovertin B were corrected for this water content (see Table II). The values are 38,700(369), 31,000(273.5), 30,900(268.5), and 9000(217) liter · mol^{-1} · cm^{-1} (wavelength in nm).

This aurovertin B sample was then dried more rigorously over phosphorus pentoxide at 50–55°C for 10 h *in vacuo* and then stored at 20°C *in vacuo*. The ^{1}H-NMR spectrum of this sample was obtained at two different concentrations in [^{2}H]$CHCl_3$ (see Fig. 2, trace B, for lower-concentration spectrum). It can be seen from the data in Table II that the water content was reduced to 0–0.35 mol/mol of aurovertin B. From the UV spectrum new ϵ values were calculated at the absorption maxima, using the molecular weight of anhydrous aurovertin B. This gave ϵ = 38,500 liter · mol^{-1} · cm^{-1} for the long-wavelength peak at 369 nm, which agrees well with the ϵ value for the earlier aurovertin B calculated for the molecular weight of the aurovertin B trihydrate sample.

It is of interest that the signal due to the 7-OH proton appears in the NMR spectrum of dry aurovertin B (see Fig. 2) at δ1.72 ppm as a doublet overlapping the multiplet due to the nonequivalent methylene protons at C-2 and coupled to the proton at C-7 (δ3.30 ppm), which now appears as a triplet rather than the doublet observed in the hydrated aurovertin B spectrum (see Fig. 1). This observation suggests that in the case of aurovertin B · $3H_2O$ (Fig. 1) the water molecules remain tightly bound to a site on the aurovertin molecule even when dissolved in [^{2}H]$CHCl_3$ such that exchange of the 7-OH proton with these water molecules is rapid on the NMR time scale. In the dry sample (Fig. 2) there is no exchange of the 7-OH proton with the very small amount of water present in the [^{2}H]$CHCl_3$ solvent.

Estimation of the Molar Absorption Coefficient of Aurovertin A

Aurovertin A was recrystallized from absolute ethanol and dried at 20°C for 6 h *in vacuo* over silica gel. By ^{1}H-NMR spectroscopy as above (data not

Table II. Definitive UV Absorption Data for the Aurovertins[a]

Aurovertin sample	mp[b] (°C) (solvent of crystallization)	Equivalents of water in crystalline sample	Molecular formula (molecular weight) used for ϵ calculation	UV absorption data[c] ϵ in liter · mol^{-1} · cm^{-1} (λ_{max} in nm)			
B	164–166 (MeOH)	3.0 3.3	$C_{25}H_{32}O_8 \cdot 3H_2O$ (514.6)	38,700 (369)	31,000 (273.5)	30,900 (268.5)	9000 (217)
B (dried rigorously)	163–164.5 (MeOH)	0 0.3	$C_{25}H_{32}O_8$ (460.5)	38,500 (369)	31,000 (273.5)	31,200 (268.5)	— —
A	233–234.5 (EtOH)	0.5 0	$C_{27}H_{34}O_9$ (502.6)	38,500 (367.5)	32,200 (237)	34,700 (267.5)	9800 (216)
D	100–144 (Et_2O)	0.6[d] 0.5[d]	$C_{25}H_{32}O_9$ $0.5H_2O$ $0.2Et_2O$ (500.3)	36,800 (369)	30,000 (273)	29,000 (268.5)	10,500 (215)

[a]The ^{1}H-NMR spectra shown in Figs. 1 and 2 and in unpublished data were analyzed for solvent content in the sample as described in the text.
[b]Uncorrected (Kofler block).
[c]Obtained for solutions in absolute ethanol.
[d]0.2 mol of diethyl ether was also detected in this sample.

shown), it was shown that there was no water or other solvent of crystallization present.

As shown in Table II the ϵ value at 367.5 nm was 38,500 liter · mol^{-1} · cm^{-1}, agreeing very well with that for aurovertin B.

Estimation of the Molar Absorption Coefficient of Aurovertin D

Aurovertin D was purified by preparative HPLC and recrystallized with difficulty from diethyl ether. Although the sample was apparently pure by HPLC with detection at 369 and 216 nm, the melting point was unsatisfactory (see Table II). The only impurities detectable by ^{1}H-NMR (spectrum not shown) were small amounts of diethyl ether and water (0.2 and 0.5 mol/mol of aurovertin D, respectively), even after drying *in vacuo* for 16 h at 50–53°C over P_2O_5. The poor melting point suggests that a small amount of a tightly associated impurity such as silicone oil or grease was still present.

Aurovertin D seems to be peculiarly difficult to purify to the extent where clean crystals with sharp melting points can be obtained readily. Bertina (1972) records similar problems. For this reason aurovertin B is preferred for precise experimentation.

The ϵ value for aurovertin D taking into account the residual solvents was 36,800 liter · mol^{-1} · cm^{-1} at 369 nm (see Table II). The profile of the UV spectrum for aurovertin D was identical with that for aurovertin B (data not shown), and so the slightly low ϵ value for our aurovertin D sample in comparison with those for aurovertins A and B is probably due to the small amount of impurity.

Discussion

The experimental results have shown unequivocally that the correct value for the molar absorption coefficient of the aurovertins is 38,500 liter · mol^{-1} · cm^{-1} for the high-wavelength absorption maximum at 369 nm in ethanolic solution (367.5 nm for aurovertin A). It is probable that this is also the correct ϵ value for the recently discovered antibiotic asteltoxin (Kruger *et al.*, 1979), which was reported to have $\epsilon_{367\ nm}$ = 32,760 liter · mol^{-1} · cm^{-1}, since the chromophore for asteltoxin is very similar to that of the aurovertins. The low values for ϵ reported in Table I were probably due to the lack of appreciation of tightly bound solvent molecules in crystals of the aurovertins and possibly in some cases to the presence of other impurities.

Solvent and water bind very tightly to aurovertins B and D, but not to aurovertin A since the 7-hydroxyl group is acetylated in aurovertin A. These observations suggest that the free 7-hydroxyl group is involved in the tight-binding interaction. The high affinity of the aurovertins for water may

relate to the mechanism of inhibition of ATPase and ATP-synthetase activities by the aurovertins. Aurovertin A is much less effective than aurovertins B and D as an inhibitor (Linnett and Beechey, 1979).

It is remarkable that the reported stoichiometries of binding of aurovertin D to a variety of ATPases under different conditions by fluorescence enhancement have been close to 1.0 (Chang and Penefsky, 1973) or to 2.0 mol aurovertin/mol ATPase (Chang and Penefsky, 1973; Muller *et al.*, 1977; Verschoor *et al.*, 1977; Stutterheim *et al.*, 1980; Wise *et al.*, 1981). Since the majority of these experiments used an ϵ value of 28,500–29,000 liter $\cdot$ mol^{-1} $\cdot$ cm^{-1}, the stoichiometry of 2.0 should be altered to about 1.5 when the correct ϵ value is used. Of course, stoichiometry measurements will also depend upon the method for measurements of the amount of protein and the molecular weight of the ATPase, as has been pointed out before (see Verschoor *et al.*, 1977).

Doubt must also be cast on the use of the fluorescence enhancement observed when aurovertin is bound to the ATPase as a method for measuring stoichiometry. The methodology of Chang and Penefsky (1973), which has been used subsequently by most other workers, assumes tacitly that the amount of flourescence enhancement reflects the amount of aurovertin bound to the ATPase. The changes in fluorescence caused by adding nucleotides (Chang and Penefsky, 1973) can also be interpreted as changes in fluorescence quenching of the same amount of the aurovertin bound to the ATPase caused by a conformational change in the binding site. The nonlinear double reciprocal plots of fluorescence enhancement and ATPase concentration at fixed aurovertin concentrations and the curved Scatchard plots observed by Muller *et al.* (1977) emphasize the problems associated with the fluorescence method and have led to complicated kinetic interpretations (Muller *et al.*, 1977). The apparent need for such complicated interpretations may lie in variations of the fluorescence yield of the different conformers of the aurovertin–ATPase complex and not in the stoichiometry of binding.

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