Cucurbitacin Δ^{23} -reductase from the fruit of *Cucurbita maxima* var. Green Hubbard

Physicochemical and fluorescence properties and enzyme–ligand interactions

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Cucurbitacin Δ^{23} -reductase from *Cucurbita maxima* var. Green Hubbard fruit displays an apparent M_r of 32000, a Stokes radius of 263 nm and a diffusion coefficient of $8.93 \times 10^{-7} \text{ cm}^2 \cdot \text{s}^{-1}$. The enzyme appears to possess a homogeneous dimeric quaternary structure with a subunit M_r of 15000. Two tryptophan and fourteen tyrosine residues per dimer were found. Emission spectral properties of the enzyme and fluorescence quenching by iodide indicate the tryptophan residues to be buried within the protein molecule. In the pH range 5–7, where no conformational changes were detected, protonation of a sterically related ionizable group with a pK of approx. 6.0 markedly influenced the fluorescence of the tryptophan residues. Protein fluorescence quenching was employed to determine the dissociation constants for binding of NADPH (K_d 17 μM), NADP⁺ (K_d 30 μM) and elaterinide (K_d 227 μM). Fluorescence energy transfer between the tryptophan residues and enzyme-bound NADPH was observed.

INTRODUCTION

The cucurbitacins, a group of tetracyclic triterpenes having a cucurbitane skeleton characterized by a 19(10 $\rightarrow$ 9 β)-abeo-10-lanostane, occur in the Cucurbitaceae and other plant families (Lavie & Glotter, 1971; Dreyer & Trousdale, 1978; Kupchan *et al.*, 1978; Hylands & Mansour, 1982). Extensive chemical studies on this group of compounds have been performed (Vande Velde & Lavie, 1983; Lavie & Glotter, 1971). Cucurbitacins have been reported to behave as anti-gibberellins (Guha & Sen, 1973; West, 1980), feeding inhibitors (Nielsen *et al.*, 1977) and insect attractants (Chambliss & Jones, 1966). Clinical interest in the cucurbitacins derives from their cytotoxic and carcinostatic properties (Suffness & Douros, 1979; Cassady & Suffness, 1980; Witkowski & Konopa, 1981; Witkowski *et al.*, 1948).

Cucurbitacin Δ^{23} -reductase (23,24-dihydrocucurbitacin:NAD(P)⁺ Δ^{23} -oxidoreductase, EC 1.3.1.5) has been proposed to play an important role in the metabolic transformation and breakdown of the cucurbitacins in (*Cucurbita maxima*) var. Green Hubbard fruit. (Schabort & Teijema, 1968; Schabort *et al.*, 1968). The enzyme catalyses the NAD(P)H-dependent reduction of the Δ^{23} bond in the side chain of cucurbitacins, for example, the conversion of elaterinide (I), the β -D-glucoside of

cucurbitacin E, into 23,24-dihydroelaterinide. Cucurbitacin Δ^{23} -reductase purified from *Cucurbita maxima* (Schabort *et al.*, 1968) is characterized by a broad substrate specificity and requires bivalent cations, notably Mn²⁺, for activity. Higher activities towards the more-water-soluble β -D-glucoside derivatives of the corresponding cucurbitacins have also been observed (Schabort & Potgieter, 1968). The purpose of the present study was to obtain information regarding the molecular and binding properties of cucurbitacin Δ^{23} -reductase from *Cucurbita maxima*.

EXPERIMENTAL

Materials

Elaterinide, the β -D-glucoside of cucurbitacin E, was a gift from Dr. P. R. Enslin of the National Research Laboratories, C.S.I.R., Pretoria, South Africa. NADPH and NADP⁺ were from Boehringer-Mannheim, Mannheim, Germany. The concentrations of elaterinide in methanol and NADPH in water were determined spectrophotometrically with the ϵ_{335} value of 11 750 $\text{M}^{-1} \cdot \text{cm}^{-1}$ (Enslin *et al.*, 1956) and the ϵ_{340} value of 6220 $\text{M}^{-1} \cdot \text{cm}^{-1}$ respectively. Cucurbitacin Δ^{23} -reductase was isolated from the fruit of *Cucurbita maxima* and purified as previously described (Schabort *et al.*, 1968). Protein concentration was determined by the biruet method (Scoffone & Fontana, 1975).

Analytical methods

Analytical gel chromatography was performed on a column of Sephadex G-200 (Andrews, 1970), which was calibrated with cytochrome *c* (M_r 12 500), chymotrypsinogen (M_r 25 000), ovalbumin (M_r 45 000), bovine serum albumin (M_r 68 000) and aldolase (M_r 158 000).

Polyacrylamide-gel electrophoresis in the absence of sodium dodecyl sulphate was performed on 7.5%-(w/v)-acrylamide gels as described by Gabriel (1971). Poly-

acrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate was performed by the method of Laemmli (1970), whereas the M_r calculations were performed as described by Weber *et al.* (1972). Isoelectric focusing over the pH range 3–10 in 1 mm-thick polyacrylamide gels was performed as described (Pharmacia Fine Chemicals, 1982).

Amino acid analyses were performed on enzyme hydrolysed in 6 M-HCl for 24 h at 110 °C and analysed on a Beckman model 120B amino acid analyser. Threonine and serine values were corrected for destruction during acid hydrolysis, and values for valine and isoleucine were corrected for incomplete liberation (Reeck, 1970). Tryptophan was determined spectrophotometrically with *p*-dimethylaminobenzaldehyde (Scoffone & Fontana, 1975).

Fluorescence measurements

Fluorescence was measured with a Perkin-Elmer MPF-3L spectrofluorimeter equipped with a thermostatically controlled sample compartment maintained at 15 °C. Excitation and emission bandwidths were 5 nm and 10 nm respectively. All fluorescence measurements were corrected for dilution and inner-filter effects (Levine, 1977).

For fluorescence quenching with iodide, successive aliquots of 4 M-KI were added to an enzyme solution in 10 mM-maleic acid/NaOH, pH 6.6, containing 0.15 M-NaCl. $\text{Na}_2\text{S}_2\text{O}_3$ (0.1 mM) was added to the KI solution to suppress I_3^- formation. Quenching data were analysed according to eqn. (1), the Stern-Volmer equation (Lehrer & Leavis, 1978), and also according to the modified Stern-Volmer relationship (eqn. 2) derived by Lehrer (1971):

$$\frac{F_0}{F} = 1 + K_q Q \quad (1)$$

$$\frac{F_0}{\Delta F} = \frac{F_0}{F_0 - F} = \frac{1}{f_{a(\text{eff})}} + \frac{1}{f_{a(\text{eff})} K_{q(\text{eff})} Q} \quad (2)$$

where F_0 and F are the fluorescence intensities in the absence and presence of quencher, Q , respectively, and K_q the quenching constant. The $f_{a(\text{eff})}$ and $K_{q(\text{eff})}$ terms in eqn. (2) (i.e. 1/intercept and intercept/slope for $F_0/\Delta F$ -against- Q^{-1} plots respectively) are the effective fraction of quenchable fluorescence and the effective quenching constant respectively.

Fluorescence quenching induced by the binding of the ligands elaterinide, NADPH or NADP⁺ were performed by the addition of aliquots of stock elaterinide or coenzyme solutions to enzyme in 10 mM-maleic acid/NaOH, pH 6.6, containing 0.15 M-NaCl. Methanol up to a final concentration of 2.5% (v/v) had very little effect on the fluorescence of cucurbitacin Δ^{23} -reductase. Quenching data were analysed according to a rewritten form of eqn. (2):

$$\frac{F_0}{\Delta F} = \frac{F_0}{\Delta F_s} + \frac{F_0 K_d}{\Delta F_s L} \quad (3)$$

which is the Klotz (Klotz *et al.*, 1946) type of binding equation. Here F_0 and F are the fluorescence intensities in the absence and presence of ligand, L , respectively ($\Delta F = F_0 - F$), ΔF_s is the extrapolated value for ΔF for complete saturation of the ligand-binding sites, and K_d is the apparent dissociation constant (i.e. slope/intercept for $F_0/\Delta F$ -against- L^{-1} plots).

Fig. 1. Polyacrylamide-gel electrophoresis of cucurbitacin Δ^{23} -reductase

(a) Electrophoresis in 7.5%-polyacrylamide gels in Tris/glycine, pH 8.9, at 6 mA per gel. (b) Electrophoresis in 12.6%-polyacrylamide gel slabs in the presence of sodium dodecyl sulphate. Gels were stained with Coomassie Blue R-250.

RESULTS AND DISCUSSION

Cucurbitacin Δ^{23} -reductase was initially judged to be homogeneous by paper electrophoresis (Schabert *et al.*, 1968). Homogeneity was confirmed in the present study by electrophoresis on polyacrylamide gels in the absence and presence of sodium dodecyl sulphate, which yielded a single stained protein band (Fig. 1). Furthermore, these results also indicate the presence of a single enzyme form isolated from *Cucurbita maxima*. Attempts to determine a pI value for cucurbitacin Δ^{23} -reductase on isoelectric-focusing gels indicated a value of greater than 10. The amino acid composition data in Table 1 indicates a high ammonia content. Assuming that the ammonia is present in the amide form (i.e. asparagine and glutamine), the combined aspartic acid and glutamic acid content of the enzyme would be reduced from 67 to 8 residues. This would increase the net positive charge of the enzyme due to the lysine and arginine residues and offers an explanation for the high pI value observed.

Molecular properties

The M_r , Stokes radius and diffusion coefficient of the enzyme were determined by means of analytical gel chromatography. An apparent M_r value of 32000, a Stokes radius of 263 nm and a diffusion coefficient of $8.93 \times 10^{-7} \text{ cm}^2 \cdot \text{s}^{-1}$ were obtained. The last value, when used in conjunction with the $s_{20,w}^0$ value of 2.96 S obtained from sedimentation-velocity experiments (Schabert *et al.*, 1968) gave a M_r value of 30000, which is in agreement with that obtained from gel filtration.

Table 1. Amino acid composition of cucurbitacin Δ^{23} -reductase from *Cucurbita maxima*

Tryptophan was determined spectrophotometrically with *p*-dimethylaminobenzaldehyde and cysteine as *S*-carboxymethylcysteine. Corrections were made for threonine, serine, valine and isoleucine (Reeck, 1970). An M_r value of 30000 was used in the calculation of amino acid residues per molecule of enzyme. Abbreviation used: ND, not detected.

Amino acid	Composition (residues/molecule)
Asx	37
Thr	20
Ser	23
Glx	30
Pro	16
Gly	26
Ala	28
Cys	ND
Val	19
Met	6
Ile	10
Leu	25
Tyr	14
Phe	13
Lys	10
His	4
Arg	18
Trp	2
NH ₃	59

Also, by employing the $s_{20,w}^0$ value of 2.96 S, a frictional ratio (f/f_0) value of 1.13 was calculated, which indicates the globular character of the enzyme.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis yielded a single protein band (Fig. 1b) that corresponds to a subunit M_r value of 15000. Comparison with the M_r value of 32000 obtained from gel filtration suggests that the native reductase possesses an apparently homogeneous dimeric quaternary structure.

Intrinsic fluorescence properties

Cucurbitacin Δ^{23} -reductase contains two tryptophan and fourteen tyrosine residues per molecule (Table 1). Upon excitation at 295 nm, where tyrosine does not absorb light appreciably, the reductase enzyme exhibited an emission maximum at 330 nm. The emission properties of the enzyme, such as the position of the emission maximum and half-bandwidth (53 nm) of the emission spectrum, are typical for a protein containing tryptophan residues buried within the protein molecule (Longworth, 1971; Burnstein *et al.*, 1973). The position of the emission maximum and the shape of the spectrum did not change as the excitation wavelength was varied from 278 nm to 295 nm. On excitation at 278 nm, the tyrosine residues contributed very little to the total fluorescence. Increasing the ionic strength from 0 to 0.24 M with NaCl had no effect on the fluorescence properties of cucurbitacin Δ^{23} -reductase.

A marked dependence of the fluorescence intensity at 330 nm on pH was observed, as shown in Fig. 2. The pH drift over the pH range 5–8 was small (< 0.1 pH unit). No change in the emission maximum was observed,

Fig. 2. pH-dependence of the fluorescence intensity at 330 nm of cucurbitacin Δ^{23} -reductase

The results are relative to the fluorescence at pH 6.65 ($\equiv 1$). Enzyme was $1.8 \mu\text{M}$ in 10 mM-maleic acid/NaOH containing 0.15 M-NaCl. Emission was at 330 nm and excitation at 278 nm (○) and 295 nm (●).

indicating that the enzyme did not undergo major conformational changes in the pH range 5–8. The pH-dependence of tryptophan fluorescence for a protein under conditions that exclude denaturation and conformational changes may reveal the presence of ionization-dependent quenching processes in the protein. From Fig. 2 a pK value for the ionizable group(s) controlling the enzyme fluorescence at pH values below 7 was estimated to be approx. 6. Because tryptophan fluorescence can be strongly quenched by the protonation of a histidine residue in its vicinity, as demonstrated for other proteins (Shinitzki & Goldman, 1967; Steiner, 1971), we propose a similar mechanism, assuming that only one ionizable group is involved. (Although more complicated mechanisms involving other ionizable groups can also explain this process, we have chosen the simplest mechanism consistent with the available data.) The absence of cysteine (Table 1) precludes the participation of thiol groups. Ionization of lysine and/or tyrosine may result in the dependence observed between pH 8 and 10. However, since the emission maximum shifted to the red, conformational changes cannot be excluded.

Iodide quenching studies

The exposure of cucurbitacin Δ^{23} -reductase to iodide lead to a decrease in the fluorescence intensity at 330 nm. The fluorescence spectral features and emission maxima remained unchanged over the total range of iodide used (0–0.25 M), indicating that the polarity of the tryptophan residues did not change. The downward-curved Stern–Volmer plot (Fig. 3) indicates that the quenching process cannot be explained by eqn. (1). Values for $f_{a(\text{eff})}$ and $K_{q(\text{eff})}$ calculated from the modified Stern–Volmer

Fig. 3. Stern-Volmer plot for iodide fluorescence quenching of cucurbitacin Δ^{23} -reductase

The enzyme was $1.9 \mu\text{M}$ in 10 mM-maleic acid/NaOH, pH 6.6, containing 0.15 M-NaCl. Excitation and emission were 295 nm and 330 nm respectively. The inset graph shows a modified Stern-Volmer plot of the same data.

equation (eqn. 2; Fig. 3 inset) are 0.4 and 4.41 M^{-1} respectively. Iodide is, therefore, a weak quenching agent of the tryptophan residues in the reductase enzyme. Furthermore, the observation that only 40% (i.e. $f_{a(\text{eff})} \times 100$) of the tryptophan fluorescence was accessible to quenching by iodide, indicates that the tryptophan residues are partially buried within the protein molecule. Iodide has been shown to quench preferentially the external tryptophan residues of proteins (Lehrer, 1971; Eftink & Ghiron, 1976).

Ligand-binding properties

Quenching of the intrinsic protein fluorescence at 330 nm was employed to observe the binding of elaterinide, NADPH and NADP^+ to cucurbitacin Δ^{23} -reductase. No change in the emission was observed. By plotting the quenching data according to eqn. (3), as shown in Fig. 4, dissociation constants (i.e. slope/intercept values for the $F_0/\Delta F$ -against- $[L]_{\text{free}}^{-1}$ plots) were estimated to be approx. $17 \mu\text{M}$ for NADPH, $30 \mu\text{M}$ for NADP^+ , $227 \mu\text{M}$ for elaterinide and $32 \mu\text{M}$ for NADP^+ in the presence of $23 \mu\text{M}$ -elaterinide. Maximum fluorescence quenching values of 36, 14 and 34% were calculated for NADPH, NADP^+ and elaterinide respectively. A value of 10% was obtained for NADP^+ in the presence of elaterinide. The results indicate NADPH to bind approx. 1.8-times more strongly than the oxidized coenzyme.

The quenching of the intrinsic protein fluorescence by NADPH probably results from the transfer of excitation energy from the tryptophan residues to NADPH (Föster, 1948), since the emission spectrum of the enzyme overlaps the absorption spectrum of NADPH. The fluorescence of enzyme-bound NADPH was enhanced and its emission maximum blue-shifted from 454 nm to 445 nm. The excitation difference spectrum due to the enzyme-NADPH

Fig. 4. Klotz binding plots for ligand binding to cucurbitacin Δ^{23} -reductase

The plots are for the binding of NADPH ($\circ$), NADP^+ ($\bullet$), NADP^+ in the presence of $22.5 \mu\text{M}$ -elaterinide ($\square$) and elaterinide ($\blacksquare$) to the enzyme ($1.9 \mu\text{M}$) in 10 mM-maleic acid/NaOH, pH 6.6, containing 1 mM- MnCl_2 and 0.15 M-NaCl. Excitation and emission were at 295 nm and 330 nm respectively.

complex demonstrated a large excitation maximum at 290 nm, as well as at 345 nm, evidently arising from the transfer of energy from the tryptophan residues to bound NADPH (Velick, 1958). The dependence of an activation peak at 290 nm on protein rather than on the simple enhancement of absorbance of the adenine moiety of NAD(P)H has been confirmed (Velick, 1958; Summers & Yielding, 1970). Since elaterinide and NADP^+ cannot quench tryptophan fluorescence by energy transfer, the decrease in fluorescence may result either from a conformational change, which alters the local environment of the tryptophan residues, or from a change in the fluorescence lifetimes of the tryptophan residues. The insensitivity of the emission maximum to either elaterinide or NADP^+ binding would appear to preclude the former possibility.

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REFERENCES

- Andrews, P. (1970) *Methods Biochem. Anal.* **18**, 1-53
- Burnstein, E. A., Vadenkina, N. S. & Ivkova, M. N. (1973) *Photochem. Photobiol.* **18**, 263-279
- Cassady, J. M. & Suffness, M. (1980) *Med. Chem.* **16**, 201-269
- Chambliss, O. L. & Jones, C. M. (1966) *Science* **153**, 1392-1393
- Dreyer, D. L. & Trousdale, E. K. (1978) *Phytochemistry* **17**, 325-326
- Eftink, M. R. & Ghiron, C. A. (1976) *Biochemistry* **15**, 672-680

- Enslin, P. R., Joubert, F. J. & Rehm, S. (1956) *J. Sci. Food Agric.* **7**, 646–655
- Föster, T. (1948) *Ann. Phys.* **2**, 55–75
- Gabriel, O. (1971) *Methods Enzymol.* **22**, 565–578
- Guha, J. & Sen, S. P. (1973) *Nature (London)* **244**, 223–224
- Hylands, P. J. & Mansour, E.-S. S. (1982) *Phytochemistry* **21**, 2703–2707
- Klotz, I. M., Walker, F. M. & Bivan, R. B. (1946) *J. Am. Chem. Soc.* **68**, 1486–1490
- Kupchan, S. M., Meshulam, H. & Sneden, A. T. (1978) *Phytochemistry* **17**, 767–769
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Lavie, D. & Glotter, E. (1971) *Prog. Chem. Org. Nat. Prod.* **29**, 307–362
- Lehrer, S. S. (1971) *Biochemistry* **10**, 3254–3263
- Lehrer, S. S. & Leavis, P. C. (1978) *Methods Enzymol.* **49**, 222–236
- Levine, R. L. (1977) *Clin. Chem.* **23**, 2292–2301
- Longworth, J. W. (1971) in *Excited States of Proteins and Nucleic Acids* (Steiner, R. F. & Weinryb, I., eds.), pp. 319–484, Plenum Press, New York
- Nielsen, J. K., Larsen, L. M. & Sørensen, H. (1977) *Phytochemistry* **16**, 1519–1522
- Pharmacia Fine Chemicals (1982) *Isoelectric Focusing: Principles and Methods*, pp. 49–59, Uppsala, Sweden
- Reeck, G. (1970) in *Handbook of Biochemistry* (Sober, H. A., ed.), p. C-281, CRC Press, Cleveland, OH
- Schabert, J. C. & Potgieter, D. J. J. (1968) *Biochim. Biophys. Acta* **151**, 47–53
- Schabert, J. C. & Teijema, H. L. (1968) *Phytochemistry* **7**, 2107–2110
- Schabert, J. C., Potgieter, D. J. J. & De Villiers, V. (1968) *Biochim. Biophys. Acta* **151**, 33–46
- Scoffone, E. & Fontana, A. (1975) in *Protein Sequence Determination* (Needleman, S. B., ed.), pp. 162–203, Springer Verlag, New York
- Shinitzki, M. & Goldman, R. (1967) *Eur. J. Biochem.* **3**, 139–144
- Steiner, R. F. (1971) *Biochemistry* **10**, 771–778
- Suffness, M. & Douros, J. D. (1979) *Methods Cancer Res.* **16**, 73–126
- Summers, M. R. & Yielding, K. L. (1970) *Biochim. Biophys. Acta* **223**, 374–382
- Vande Velde, V. & Lavie, D. (1983) *Tetrahedron* **39**, 317–321
- Velick, S. F. (1958) *J. Biol. Chem.* **233**, 1455–1467
- Weber, K., Pringel, J. R. & Osborn, M. (1972) *Methods Enzymol.* **26**, 3–27
- West, C. A. (1980) in *Plant Growth Substances 1979* (Skoog, F., Ed.), pp. 289–290, Springer-Verlag, Berlin
- Witkowski, A. & Konopa, J. (1981) *Biochim. Biophys. Acta* **674**, 246–255
- Witkowski, A., Woynarowska, B. & Konopa, J. (1984) *Biochem. Pharmacol.* **33**, 995–1004

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