The use of electrospray mass spectrometry to identify an essential arginine residue in type II dehydroquinases

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Abstract The arginine-specific reagent phenylglyoxal has been used to identify a hyper-reactive arginine residue which is essential for activity in the type II dehydroquinases of *Streptomyces coelicolor* and *Aspergillus nidulans*. Electrospray mass spectrometry was used both to characterise the phenylglyoxal modified protein, and to identify the phenylglyoxal modified peptides following enzymatic digestion. The advantages of using electrospray mass spectrometry for monitoring arginine modication aimed at identifying functional residues in proteins are discussed.

Key words: Type II dehydroquinase; Phenylglyoxal; Electrospray mass spectrometry

1. Introduction

The type II dehydroquinases (3-dehydroquinate dehydratases, EC 4.2.1.10) catalyse the conversion of dehydroquinate to dehydroshikimate. This reaction, which occurs on both the biosynthetic shikimate pathway and the catabolic quinate pathway [1–3], involves the *trans* elimination of water [4]. Little is known about the structure and mechanism of the type II dehydroquinases which are clearly mechanistically and structurally different from the better characterised type I enzymes [5,6]. The type I enzymes are exclusively biosynthetic [5], have a conserved active site lysine residue [7], and catalyse a *cis* elimination via an imine intermediate [4,8] with the participation of a conserved histidine residue as the general base [9]. In contrast the fungal type II enzymes have an exclusively catabolic role [2,10] while the bacterial type II enzymes may be exclusively biosynthetic [11,12] or be involved in both biosynthesis and catabolism [13]. There is preliminary chemical modification evidence for a role for histidine in the type II enzymes [5], but there are no conserved lysine residues and this, together with the different stereochemistry and the failure of experiments to inhibit the type II enzymes with substrate and sodium borohydride [5], emphasises their mechanistic distinction from the type I enzymes.

A type II dehydroquinase has recently been crystallised [7] and to facilitate the structure determination we have been using group specific chemical modification reagents to identify amino acid residues in the active site. Many enzymes which use carboxylic acids as substrates utilise an arginine residue for carboxylate recognition [14] and in several cases the functional arginine has proved to be hyper-reactive [15]. The reagent phenylglyoxal (PGO) has been widely used to demonstrate the involvement of arginine residues in enzyme function [16,17]. However, the identification of specific arginine residues is complicated by the relatively low stability of the adducts formed [17], their variable stoichiometry [17] and the necessity of using radiolabelled reagent. Also in the case of the type II dehydroquinases the identification of active site residues is further complicated by the relatively low affinity of the enzymes for both substrate and product and the lack of tight binding competitive inhibitors [5]. To determine whether an active site arginine was present a method was needed for analysing enzyme in the early stages of modification and correlating the modification of particular residues directly with the loss of activity. We have used electrospray mass spectrometry to monitor the reaction of two type II dehydroquinases with PGO. This has permitted the simple characterisation of singly and multiply modified enzyme molecules and, through the direct analysis of enzymatic digests of the modified enzyme, the direct location of the modification sites without the necessity for sequencing or the use of radioactive or other labels. By this means we have identified a single hyper-reactive arginine residue that is essential for activity and is probably in the substrate binding site of the type II dehydroquinases.

2. Experimental

The type II dehydroquinases from *Streptomyces coelicolor* [11] and *Aspergillus nidulans* [18] were overexpressed in *Escherichia coli* and assayed [5] and purified as described previously [19,18]. Protein concentrations were determined spectrophotometrically at 280 nm using extinction coefficients calculated from the amino acid compositions [20].

Enzyme samples in 100 mM sodium bicarbonate buffer, pH 9.4 were pre-incubated for 5 min at 25°C and then PGO (freshly made up 50 mM stock solution in water) was added to a final concentration of 0.5–4.0 mM. Aliquots were removed at various times for enzyme assay. Enzyme inactivated to different extents for mass spectrometry was prepared by stopping the reaction by gel filtration on a Sephadex G50 column (200 mm × 17 mm, flow rate 15 ml·h^{-1}) equilibrated with 10 mM ammonium bicarbonate. To remove low molecular weight contaminants prior to mass spectrometry the enzyme samples were washed twice by diluting 50-fold with HPLC grade water and reconcentrated using Centricon-10 centrifugal concentrators (Amicon, Stonehouse, Gloucestershire, UK).

To prepare peptides from the native and modified enzyme protein samples were first denatured in 6 M GnHCl for 15 min and then diluted with 0.5% (w/v) ammonium bicarbonate to a GnHCl concentration of 1 M and incubated with 2% (w/w) chymotrypsin (stock solution 1 g/l chymotrypsin in 1 mM HCl) for 45 min at 25°C. The digestion was stopped by freezing the samples at −80°C.

Mass spectrometry was performed on a VG Platform quadrupole mass spectrometer fitted with a pneumatically assisted electrospray (ionspray) source and controlled via the VG MassLynx software (VG

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Abbreviations: DHQase, dehydroquinase; ESMS, electrospray mass spectrometry; GdnHCl, guanidine hydrochloide; HPLC, high performance liquid chromatography; MaxEnt, maximum entropy; PGO, phenylglyoxal.

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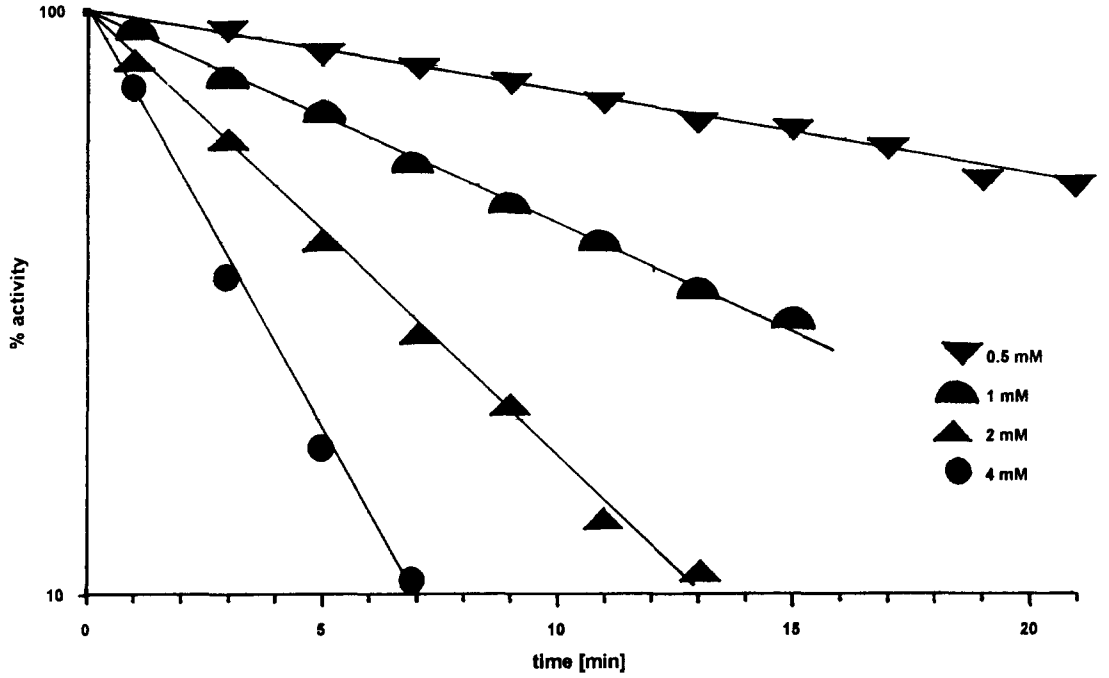

Fig. 1. Inactivation of the type II dehydroquinase of *S. coelicolor* by PGO. Semi-logarithmic plot of residual activity as a function of time. PGO concentrations 0.5 mM (▼), 1.0 mM (◓), 2.0 mM (▲) and 4 mM (●).

Biotech Ltd., Altrincham. Cheshire, UK). Carrier solvent [1:1 (v/v) acetonitrile/water, 0.2% formic acid] infusion was controlled at 10 μl/min using a Harvard syringe pump (Harvard Apparatus, South Natic, MA, USA). Protein samples were dissolved in carrier solvent at a concentration of 20 pmol/μl, centrifuged at 5000 × *g* for 2 min and then 10–20 μl samples injected directly into the carrier stream. MaxEnt deconvolution [21] was applied for quantitative analysis of the raw data using a 1.0 Da peak width and 1 Da/channel resolution.

The protein digests were separated by HPLC on a C-18 reverse phase column (μBondapak, Waters, Watford, Hertfordshire, UK) using 2% (v/v) acetonitrile in 0.1% (v/v) trifluoracetic acid as the initial solvent (flow rate 0.5 ml/min); after an 8 min wash to remove GdnHCl a linear gradient of 2–70% acetonitrile (v/v) in 0.1% (v/v) trifluoracetic acid was applied to elute the peptides. The column eluant was introduced directly into the mass spectrometer with a drying gas flow of 400 $l \cdot h^{-1}$ and the source temperature set at 100°C. The absorption profile of the eluted peptides was recorded at 214 nm and centroid mass spectra in the range 400–1800 amu were recorded at 4 s intervals.

3. Results and discussion

The inactivation of the *S. coelicolor* type II dehydroquinase with PGO followed pseudo first order kinetics (Fig. 1). A secondary plot of the observed pseudo first order rate constants against PGO concentration was linear (data not shown) and gave a second order inactivation rate constant of 89 $M^{-1} \cdot min^{-1}$. Similar results were obtained for inactivation of the *A. nidulans* enzyme by PGO; in this case the second order rate constant was 150 $M^{-1} \cdot min^{-1}$. These observations suggested that arginine residue(s) were required for the type II dehydroquinase reaction.

The relationship between the extent of inactivation and the number of arginine residues modified by PGO was monitored by mass spectrometry; the spectra observed for three samples inactivated to different extents are shown in Fig. 2. During the early stages of inactivation (exemplified by 15% inactivation) the major peak was of unmodified enzyme and the only significant modified species had a mass difference of +116

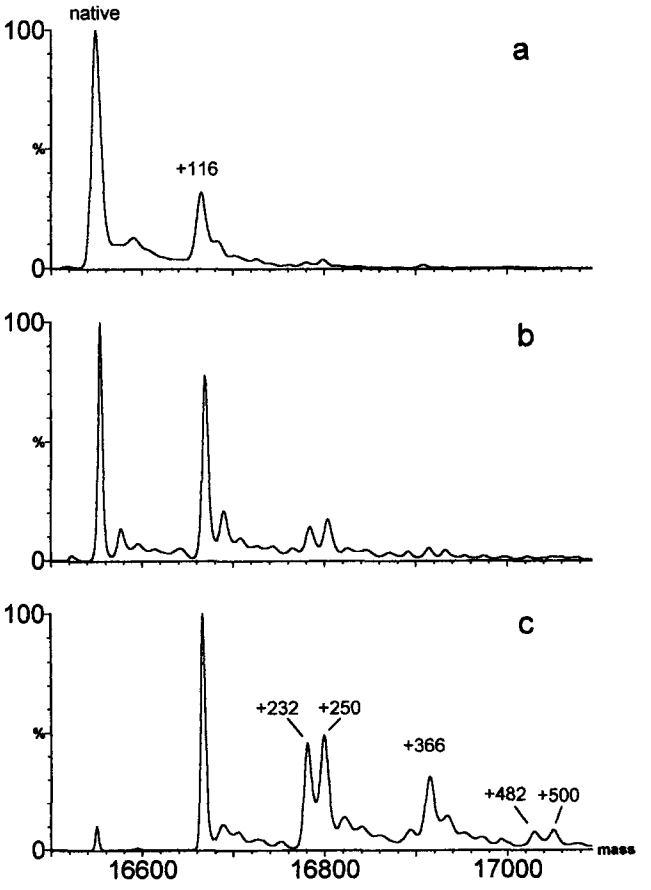

Fig. 2. Electrospray mass spectra of samples of the type II dehydroquinase of *S. coelicolor* modified to different extents with PGO: (a) 15% inactivated, (b) 60% inactivated and (c) 95% inactivated.

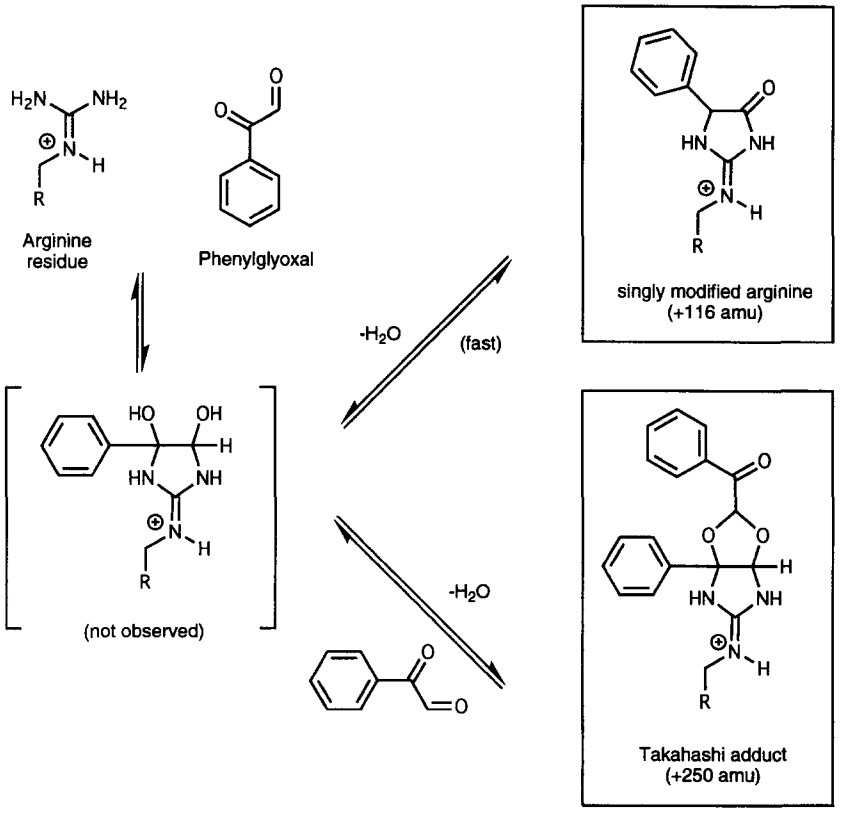

Fig. 3. Structures of adducts formed by the reaction of PGO with arginine residues.

(M_r = 16,666) (Fig. 2). This mass difference corresponds to the incorporation of one PGO per site with the loss of water (see Fig. 3). For low extents of inactivation there was a good correlation between the extent of inactivation and the relative size of this +116 peak (Table 1). In the sample that was 60% inactivated the two major peaks due to the +116 species and to unmodified protein together accounted for 80% of the material (Fig. 2). These data suggest that the initial inactivation of the enzyme is due to the formation of a simple 1:1 adduct with PGO followed by dehydration. A number of minor modified species were also present including one with a mass difference of +250 (M_r = 16,800), corresponding to the Takahashi adduct with two PGO's per arginine (see Fig. 3), and another with a mass difference of +232 (M_r = 16,782) corresponding to two different arginines, each modified with a single phenylglyoxal. These minor peaks were more significant in 95% inactivated enzyme (Fig. 2); in this sample very little unmodified protein remained, the singly modified +116 species was the most prominent peak and there were also small peaks due to multiply modified species and to Takahashi adducts for example at +232, +250, +366 (250 plus 116), +482 (250 plus 116 plus 116) and at +500 (250 plus 250).

To identify the primary site of modification samples of *S. coelicolor* dehydroquinase, modified to different extents with PGO, were digested with chymotrypsin and the peptides separated and analysed by reverse phase chromatography/electrospray mass spectrometry. In the 15% inactivated sample there was a single modified peptide of mass 3219 which corresponds to residues 1–28 of the enzyme +116 mass units. This peptide contains 2 arginine residues (R2 and R23). When the currently available type II dehydroquinase sequences are aligned to give best homology there is no conserved arginine that corresponds to R2 in the *S. coelicolor* enzyme. However, there is a totally conserved arginine that corresponds to R23 of the *S. coelicolor*

Table 1
Relative amounts of the adducts formed between PGO and the type II dehydroquinases. Samples were inactivated by treatment with PGO for various times, assayed for residual enzyme activity and then analysed by electrospray mass spectrometry (see Experimental section).

Enzyme source	% inactivation	Relative percentages of adducts						
		Native	+116	+232	+250	+366	+482	+500
S. coelicolor	15	77	23	–	–	–	–	–
	60	44	35	10	11	–	–	–
	95	2	31	19	21	18	4	5
A. nidulans	10	82	18	–	–	–	–	–
	30	66	34	–	–	–	–	–
	90	16	58	14	12	–	–	–

enzyme. This is R19 of the *A. nidulans* enzyme. It therefore seemed likely that R23 was the site of initial modification in the *S. coelicolor* enzyme. Consistent with this observation *A. nidulans* enzyme modified in a similar way was found to contain a peptide of mass 2852 which corresponds to residues 1–24 of the enzyme plus 116 mass units; this peptide contains the single arginine residue R19 which corresponds to R23 in the *S. coelicolor* enzyme.

In samples of 80% inactivated *S. coelicolor* enzyme the singly modified peptide 1–24 was found and, in addition, singly modified peptides corresponding to residues 84–116 (R113) and 139–156 (R144 and R155) were also observed. At this extent of modification it was not possible to detect any peptides containing the Takahashi adduct. The *A. nidulans* enzyme contains no arginine residues corresponding to R144 and R155. It does contain a residue corresponding to R113 but even at 95% inactivation no modification at this site was observed. The type II dehydroquinases contain an additional conserved arginine residue (R117 in the *S. coelicolor* enzyme). No evidence that this residue is modified by PGO has been obtained, but it should be noted that peptides containing this residue have proved difficult to detect by mass spectrometry.

These results suggests that the conserved residue R23 in the *S. coelicolor* enzyme (R19 in the *A. nidulans* enzyme) is essential for enzyme function. This arginine is clearly hyper-reactive and on this basis is very likely to be involved in binding the carboxylate group of the substrate as has been shown for hyper-reactive arginine residues found at the carboxylate binding sites of other enzymes [15]. Much of the earlier work on arginine modification of enzymes with PGO has assumed that the major initial reaction involved the formation of the 2:1 Takahashi adduct [17] and the extent of modifcation was usually estimated by monitoring the incorporation of radioactive PGO and assuming this 2:1 stoichiometry. Our results show that the initial PGO modification of the type II dehydroquinases does not involve formation of the 2:1 Takahashi adduct but instead a 1:1 adduct forms which is then very rapidly dehydrated (Fig. 3). Our observations also confirm that the adducts formed between PGO and proteins are sufficiently stable to be analysed by the standard conditions used for the electrospray mass spectrometry of proteins. A major difficulty in locating the sites of PGO modification has been the poor stability of the PGO modified peptides during purification, especially during reverse phase hplc in the presence of 0.1% acid. Although, under the conditions we describe there is undoubtedly significant hydrolysis of the PGO peptide adducts, the high sensitivity of electrospray mass spectrometry permits the simple and rapid identification of the major sites of arginine modification in proteins.

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