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Oxidation of rat insulin II, but not I, leads to anomalous elution profiles upon HPLC analysis of insulin-related peptides

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Rat insulin II, unlike rat insulin I and other non-rodent insulins, contains a unique methionine residue at position B29. Reversed-phase HPLC allows for separation of the two rat insulins, with insulin I typically eluting faster than insulin II. An anomalous peak of insulin immunoreactive material was found eluting even faster than insulin I following acid extraction of rat insulin-producing cells. This early peak co-eluted with [Met-O^{B29}]insulin II suggesting that during cell extraction and subsequent purification steps, rat insulin II is subject to selective oxidation at Met^{B29}. Such oxidation of rat proinsulin II affords improved separation from rat proinsulin I compared to the native form.

Insulin biosynthesis; HPLC; Methionine sulfoxide; (Rat)

1. INTRODUCTION

Reversed-phase HPLC is the method of choice for the analysis of insulin and related peptides and has allowed for identification of mutant human insulins [1] and for the separation of proinsulin and its conversion intermediates [2]. This analytical method has allowed for separation of the protein products of the two non-allelic rat insulin genes, rat insulins I and II [3–6] and rat C-peptides I and II [7]. The separation of rat proinsulin I and II by HPLC proved not to be as successful [3,6].

We have recently succeeded in transfecting cells of a corticotroph line (AtT20 cells) with the rat insulin II gene [8]. These cells were found to produce and secrete immunoreactive insulin. When the cells were extracted and insulin immunoreactive products analysed by HPLC, we detected not only proinsulin II and insulin II but also a third prominent peak of immunoreactive material eluting rapidly from the HPLC system. Although initially thought to be a proinsulin conversion intermediate [8] (possibly mono- or diarginyl insulin), it became apparent that the kinetics of the production of this material, and its abundance relative to that of proinsulin and insulin, were not consistent with this assignment. The present study provides evidence that this material is the sulfoxide form of rat insulin II ([Met-O^{B29}]insulin II), suggesting that the extraction and pre-purification techniques used by us and others prior to HPLC analysis of insulin related peptides, favour selective oxidation of Met^{B29} of rat insulin II. Taking this observation into account, the interpretation of the HPLC profiles becomes straightforward, and, furthermore, the oxidation of rat proinsulin II allows for its separation from rat proinsulin I much more readily than for the native form.

2. MATERIALS AND METHODS

2.1. *Biosynthetic labelling of rat proinsulins and insulins*

Islets of Langerhans were isolated from the pancreas of adult male Sprague-Dawley rats by a modification of the collagenase

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digestion method [9] and islets separated from exocrine tissue by the use of Histopaque 1083 (Sigma, St Louis, MO, USA). After 24 h in tissue culture (DMEM, 10% newborn calf serum, 8.3 mM glucose) the islets were labelled for 1 h at 37°C in Krebs Ringer bicarbonate buffer containing 10 mM Hepes, 0.1% (w/v) bovine serum albumin, 16.7 mM glucose and either 1 mCi/ml [^{3}H]leucine or [^{35}S]methionine (both from Amersham International, Amersham, Bucks, England). After washing twice in the above buffer without labelled amino acids and containing 2.8 mM glucose, the islets were incubated for up to 2 h in the wash buffer to allow for conversion of radioactively labelled proinsulin to insulin. Labelled products were extracted from the islets by sonication on ice in 0.2 M glycine/0.1% BSA, pH 8.8. Extracts from islets harvested at selected times of the chase incubation following labelling with either [^{3}H]leucine or [^{35}S]methionine were mixed together in order to obtain a solution with radioactivity distributed in roughly equal amounts between [^{3}H]insulin I; [^{3}H]proinsulin I; [^{3}H]proinsulin II; [^{35}S]insulin II; [^{35}S]proinsulin II.

2.2. *Extraction of transfected AtT20 cells and pre-purification of proinsulin/insulin*

AtT20 cells were transfected with the rat insulin II gene as described previously ([8] and manuscript in preparation). Stable transfectants (based upon ability to grow in the presence of the antibiotic G418) were screened for insulin immunoreactivity and positive clones then expanded in tissue culture. After washing in PBS, cells were extracted in 5 M acetic acid, 0.1% BSA. The acid extracts were freeze-thawed thrice and then sonicated. Insulin and proinsulin were then concentrated by adsorption and selective elution from a C18 Sep-Pak cartridge (Waters Associates, Milford, MA, USA) [10]. The eluted products were lyophilised and then dissolved in 0.5% trifluoroacetic acid.

2.3. *Reversed phase HPLC*

A Beckman System Gold consisting of a model 126 programmable solvent module and a model 166 programmable detector module was used with a Beckman Ultrasphere ODS 5 μm column (4.6×250 mm). The two buffer systems used were: (A) TEAP (50 mM phosphoric acid, 20 mM triethylamine, 50 mM sodium perchlorate, adjusted to pH 3.0 with NaOH); (B) 90% acetonitrile, 10% water (v/v). Insulin and related peptides were eluted using a modification of our previous methods [3,4]. This modification allowed for better separation of rat proinsulin I from II, particularly following oxidation of proinsulin II. The two rat insulins were eluted isocratically at 34.5% B. At 25 min the composition of the elution mixture was changed to 37% B over a 60 min period using a convex curve for increasing the B component ('curve 3' of the Beckman System Gold). The curve is depicted in fig.2.

2.4. *Selective oxidation of MetB^{29} of rat insulin II*

Aliquots of the mixture containing the radioactively labelled rat insulins and proinsulins were acidified to pH 2.5 using 1 M perchloric acid and a solution of 35% hydrogen peroxide was then added to obtain a final concentration of 1%, or approx. 0.3 M, in peroxide. Oxidation was allowed to proceed for 1 h at room temperature. This protocol was adapted from a published procedure for the selective oxidation of methionine residues in protein [11]. The oxidised sample was then lyophilised and redissolved in 0.1% trifluoroacetic acid, 0.1% BSA before injection into the HPLC system. Controls were handled in parallel with the exception of the omission of peroxide.

2.5. *Insulin radioimmunoassay*

Insulin immunoreactivity was measured by radioimmunoassay using the charcoal-dextran separation technique [12]. Rat insulin was used as the standard (Novo Industri, Bagsvaerd, Denmark) with guinea-pig anti-insulin serum.

3. RESULTS AND DISCUSSION

AtT20 cells transfected with the rat insulin II gene were extracted in acid and the extracted products first pre-purified and concentrated using a C18 Sep-Pak. Material eluting from the Sep-Pak was lyophilised, redissolved and then applied to HPLC. The fractions eluting from the HPLC system were lyophilised and reconstituted before being assayed for insulin immunoreactivity. The elution profile for insulin immunoreactivity is shown in fig.1A. The first peak eluted at approx. 11–13 min referred to below as 'early peak' and the second at 23–26 min. An elution profile for an extract of rat islets is shown below, in fig.1B. Again two peaks are observed, but they correspond to native rat insulins I and II as indicated. As can be seen, the second of the two peaks from the transfected cells co-eluted with rat insulin II. There was, however, no immunoreactivity for islet extracts eluting at 11–13 min. It should be noted that the islets were extracted and then immediately analysed by HPLC without the intervening concentration step using the Sep-Pak cartridge. If islets were handled in parallel with transfected cells and processed identically, then immunoreactive material eluting at 11–13 min was generated at the expense of immunoreactivity associated with rat insulin II (not shown). Interestingly, this was not a reproducible finding and, furthermore, the relative amount of early peak material and native insulin II was found to vary considerably from one experiment to the next, both for transfected cells and native islets.

Taken together, these observations suggested to us that we were dealing with some change to rat insulin II arising during the extraction/purification procedure routinely used for the transfected cells. Since, in native islets, only insulin II seemed to generate early peak material, our attention was drawn to Met^{B29}, a residue unique to rat (and mouse) insulin II. In particular, we speculated that

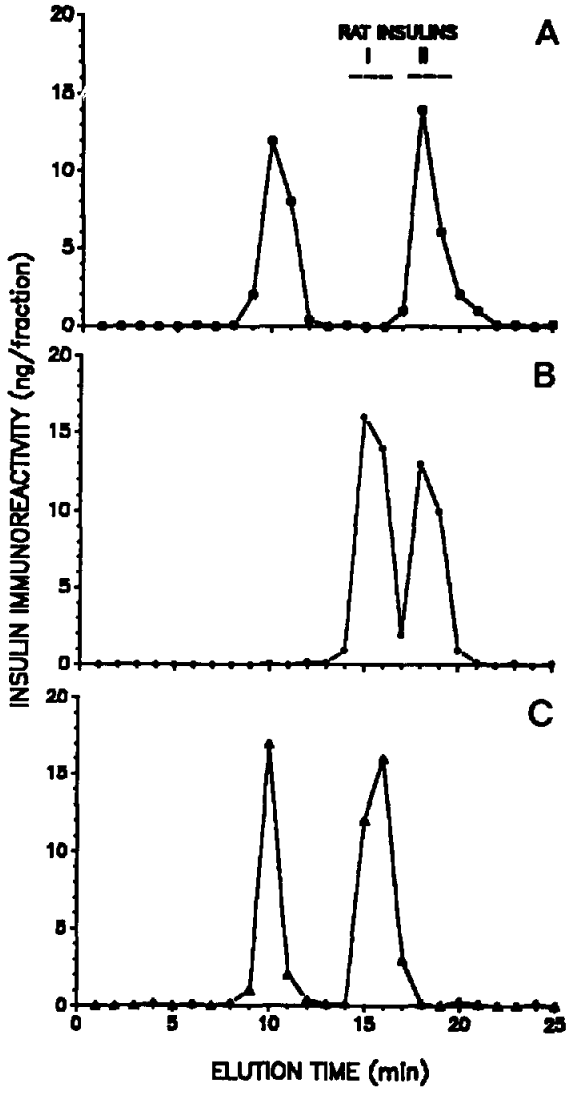

Fig.1. Insulin immunoreactivity in fractions eluting from reversed-phase HPLC under isocratic conditions (34.5%, buffer B). (A) Material extracted from AtT20 cells transfected with the gene for rat insulin II. (B) Material extracted from rat islets. (C) Material extracted from rat islets and oxidised (incubated under acidic conditions with peroxide) before analysis by HPLC. The elution times for rat insulins I and II are as indicated.

this residue may be sensitive to oxidation and especially so under acidic conditions [11,13,14]. We therefore incubated islet extracts under acidio conditions with peroxide using conditions mild enough not to oxidise any residues other than methionine [11,14]. HPLC analysis of this material is shown in fig.1C. The peroxide oxidation resulted in a complete disappearance of rat insulin II immunoreactivity in favour of an immunoreactive product eluting at 11–13 min. Rat insulin I was not affected by the peroxide treatment. The results strongly suggest that the early peak material found in the transfected cells and islets following acid extraction and prepurification by Sep-Pak is, thus, [Met-O^{B29}]insulin II.

The production of the sulfoxide form of insulin II during certain extraction procedures can clearly lead to confusing HPLC elution profiles. Indeed, we ourselves initially suspected that the early peak material in the transfected cells was a proinsulin conversion intermediate [8]. A review of the literature indicates that the problem is not unique to rat insulin II and extraction of insulin-producing cells. Thus, acid extraction of a peptide with corcitotropin-releasing activity from ovine hypothalamus led to purification of a peptide which eluted from HPLC some 5 min earlier than the chemically synthesized peptide standard and which displayed reduced biological activity [13]. Based upon the same series of experiments as described here, the authors concluded that during their extraction procedures, they were generating the methionine sulfoxide form of the peptide.

Despite the ease of separation of rat C-peptides I and II, and rat insulins I and II by HPLC [3–6], the separation of the two rat proinsulins has not proved as simple [3,6]. Based upon the dramatic change in mobility of rat insulin II following oxidation of Met^{B29}, we speculated that [Met-O^{B29}]proinsulin II may prove readily separable from both native proinsulin II and, more importantly, from rat proinsulin I. As shown in fig.2, our present convex gradient system allows for a modest separation of the two proinsulins, but they cannot be clearly resolved. The mixture of labelled insulin I and II, and proinsulins I and II was then oxidised as described earlier for islet extracts. Insulin II (labelled with [^{35}S]methionine) now eluted at 11–13 min as expected for the sulfoxide form. Native rat proinsulin II eluted at 67–69 min, whereas after its oxidation [Met-O^{B29}]proinsulin II eluted at 51–54 min, allowing for clear separation from rat proinsulin I, the elution time of which was unaffected by peroxide treatment.

In conclusion, under certain circumstances, the acid extraction of rat insulin-producing cells can

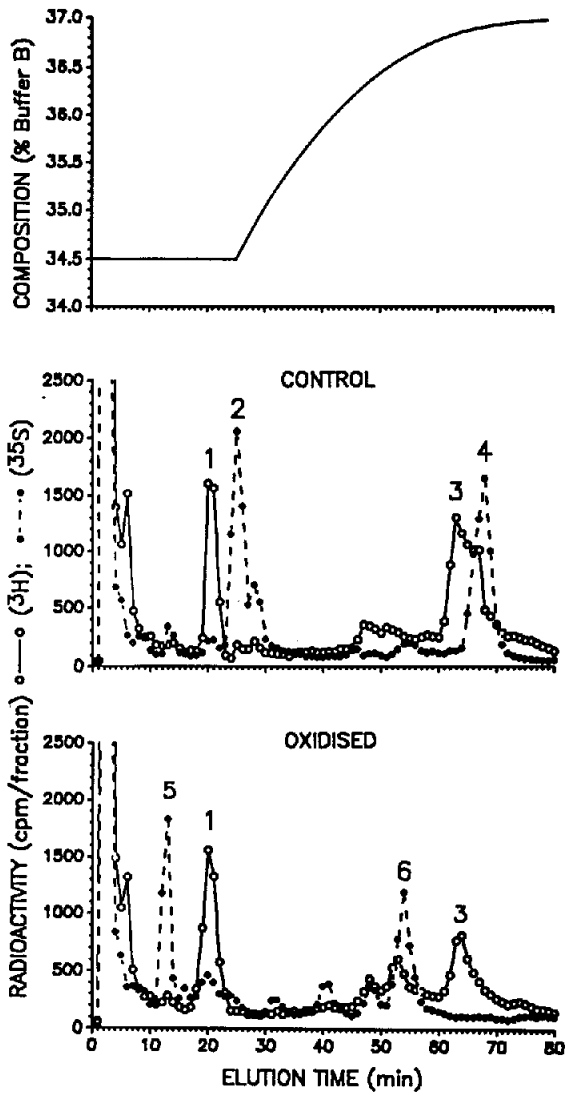

Fig.2. Effect of oxidation upon elution times of rat insulin II and proinsulin II by reversed-phase HPLC. Aliquots of a solution containing [^{3}H]rat insulin I, [^{3}H]rat proinsulin I, [^{3}H]rat proinsulin II, [^{35}S]rat insulin II and [^{35}S]rat proinsulin II, were loaded onto the HPLC system either before (control) or after (oxidised) oxidation (see legend to fig.1). The HPLC elution conditions were as follows (buffer B = 90% acetonitrile; 10% H_2O): 34.5% B for the first 25 min; increased from 34.5 to 37% B from 25 to 65 min using a convex curve as indicated in the upper panel of the figure. The identity of the numbered peaks is: (1) rat insulin I; (2) rat insulin II; (3) rat proinsulin I; (4) rat proinsulin II; (5) rat [Met-O^{B29}] insulin II; (6) rat [Met-O^{B29}] proinsulin II.

lead to selective oxidation of rat insulin II Met^{B29}. Understanding this allows for unambiguous interpretation of HPLC elution profiles during analysis of such material. Advantage can be taken of the pronounced change in elution times of rat proinsulin II vs rat [Met-O^{B29}] proinsulin II in order to separate rat proinsulin I from II by HPLC.

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