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AFFINITY TECHNIQUE FOR THE ISOLATION OF POLYPEPTIDES CONTAINING ARGININE MODIFIED WITH CYCLOHEXANE-1,2-DIONE, AND THEIR ANALYSIS BY COMBINED GAS–LIQUID CHROMATOGRAPHY–MASS SPECTROMETRY

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SUMMARY

A technique is described whereby a polypeptide containing an arginine residue that has been modified with cyclohexane-1,2-dione may be digested with a protease and any arginine-containing peptides specifically adsorbed to an affinity column consisting of immobilized borate. After desorption, the peptides may be converted into a derivative compatible with N-trifluoroacetylation and permethylation and then subjected to analysis by combined gas–liquid chromatography–mass spectrometry. Alternatively, after isolation the cyclohexanedione group may be removed and the peptide analysed by conventional procedures. Improved reaction conditions, involving use of urea, for modification with cyclohexanedione are described that were used successfully to modify insulin and a 65-residue haem-containing fragment from cytochrome-*c*. The sequence Arg–Gly–Phe was identified by mass spectrometry in a peptide isolated by affinity chromatography of a digest of cyclohexanedione-modified insulin. The methods described in this paper are appropriate both to primary structure determination and in structure–function studies via chemical modification of arginine residues.

INTRODUCTION

The analysis of partial hydrolysates of proteins and polypeptides by combined gas–liquid chromatography–mass spectrometry (GLC–MS)[1–6] offers several advantages over techniques of amino acid sequence determination that rely on the Edman degradation. To be able to exploit these advantages, the oligopeptide derivatives which it is necessary to prepare must both be volatile and possess good mass spectral characteristics. N-Trifluoroacetylation followed by permethylation is suitable and is directly applicable to peptides containing residues of any of the commonly occurring amino acids, except arginine[7]. This amino acid does not give rise to volatile products

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owing to the high basicity of its guanido group, which must therefore first be modified in a separate reaction.

The fact that arginine residues are chemically distinct from residues of the other amino acids permits their selective modification. Of the various methods available, the products of hydrazinolysis, condensation with pentane-2,4-dione and treatment with cyclohexane-1,2-dione (CHD) in sodium hydroxide solution have proved chemically compatible with the permethylation reaction involving dimethylsulphinyl sodium and methyl iodide[3,7,8]. CHD in sodium hydroxide solution has been used in conjunction with an earlier type of permethylation reaction[9]. When CHD is used in borate buffers, its selectivity for arginine residues becomes complete[10–13]. In summary, the adduct that is formed possesses a 1,2-diol function which is stabilized by borate. At low pH, the borate–diol complex dissociates and the free adduct is stable. At alkaline pH in the absence of borate, the adduct spontaneously regenerates CHD and the guanido group, a process which may be accelerated by the addition of nucleophiles such as hydroxylamine. At pH > 12, a rearrangement takes place to yield a compound containing a spiro carbon atom. Trypsin does not cleave on the C-terminal side of arginine residues modified with CHD, a feature that is useful for amino acid sequence determination, especially in view of the reversible nature of the modification (compare the utility of reversible acylation of lysine residues[14]).

Peptides modified with CHD may be isolated by the usual techniques of gel filtration, paper chromatography and electrophoresis, if necessary using a radioactively labelled reagent[15]. The ability of the 1,2-diol function to bind borate can be exploited for affinity chromatography on a column of immobilized borate. Any peptides so isolated and therefore containing such a modified arginine residue could be identified mass spectrometrically either after alkaline rearrangement to the spiro form[7] or after regeneration of the guanido group followed by one of the procedures for arginine-containing peptides, usually hydrazinolysis or condensation with pentane-1,4-dione. An advantage of direct alkaline rearrangement of the diol is that this would retain the distinction between an arginine residue that had been modified with CHD and one that had not. Alternatively, after isolation on the borate column and regeneration of the guanido group, the peptides would, of course, be amenable to sequence determination by a non-mass spectrometric method such as the Edman degradation. This paper describes some investigations concerning the formation, isolation and analysis of the adducts of CHD with various arginine-containing polypeptides.

EXPERIMENTAL

Unless otherwise specified, reagents were of analytical-reagent grade and were used without further purification. High-voltage paper electrophoresis and staining of separated peptides were as previously described[16]. Cellulose acetate electrophoresis was performed in a Shandon MMB tank on strips of cellulose acetate (Schleicher & Schüll, Dassel, G.F.R.) and using a buffer prepared by dissolving 3.1 g of boric acid in 200 ml of deionized 8 *M* urea whilst diluting with water and 1 *M* sodium hydroxide solution; the final volume was 250 ml (pH 8.0). The 3-cm wide strips drew approximately 0.05 mA at 200 V over a length of approximately 15 cm. After air drying

at 80–100°C, staining was effected with Ponceau S (0.2% in 3% acetic acid) and destaining with 3% acetic acid. Prior to acid hydrolysis for amino acid analysis, 20 μl of mercaptoacetic acid (reagent grade, Hopkin & Williams, Chadwick Heath, Great Britain) was added when required.

Preparation of the CHD adduct of porcine insulin

Boric acid (1.55 g) was dissolved in water as the pH was adjusted to 9.0 with 5 *M* sodium hydroxide solution; the final volume was 50 ml (0.5 *M*). A 1.00-g amount of CHD (Aldrich, Milwaukee, WI, U.S.A.) was dissolved in 28.6 ml of buffer and the pH was readjusted with 5 *M* sodium hydroxide solution to pH 9.0 (0.3 *M* CHD). Boric acid (1.55 g) was dissolved in deionized 8 *M* urea and the pH was adjusted to 9.0 with 5 *M* sodium hydroxide solution; the final volume was 50 ml (0.5 *M*). CHD (175 mg) was dissolved in 5.0 ml of this buffer and the pH was readjusted with 5 *M* sodium hydroxide solution to pH 9.0 (0.3 *M* CHD). Four 100-mg batches of porcine insulin (Monocomponent, Novo) were taken. To lot A were added 5 ml of water and then 5 ml of buffered CHD solution. To lot B were added 5 ml of deionized 8 *M* urea and then 5 ml of 0.5 *M* borate buffer (pH 9.0). To lot C were added 5 ml of deionized 8 *M* urea and then 5 ml of buffered CHD solution. To lot D were added 5 ml of deionized 8 *M* urea and then 5 ml of buffered CHD–urea solution. All samples were soluble and were incubated for 2 h at 37°C. Each was then rinsed with 10 ml of 30% acetic acid into a length of boiled (1% w/v sodium hydrogen carbonate solution, 15 min) 18/32 Visking dialysis tubing and dialysed at 4°C against 5 l of cold 1% acetic acid with stirring. The diffusate was changed after 3 h, a further 17.5 h and a further 5 h. The bag contents were then freeze-dried. Yields were 99–100 mg. Samples were hydrolysed for amino acid analysis. In each instance, a duplicate hydrolysis was performed in the presence of 20 μl of mercaptoacetic acid. Samples were also examined by cellulose acetate electrophoresis.

Affinity chromatography of modified insulins and control

Samples (approximately 2 mg) of the modified insulins and the unmodified control (B) were dissolved in 0.5 ml of 1% N-ethylmorpholine (Koch-Light, Colnbrook, Great Britain; redistilled before use) buffer (pH 8.0 with acetic acid) and applied to a column (bed dimensions 80 × 5.5 mm diameter) containing *m*-aminophenylboronic acid coupled to epoxy-activated Sepharose 6B[7] that had been equilibrated and was then eluted with the same buffer. Fractions of 2.5 ml were collected. After the fifth fraction had eluted, the eluent was replaced with 1% acetic acid. The UV spectra of fractions were determined.

Performic acid oxidation of insulin

A 50-mg amount of insulin (or derivative) was dissolved in 2 ml of 98% formic acid–100-volume hydrogen peroxide (9:1). After 1 h at room temperature the solution was diluted with 100 ml of water and freeze-dried to a fluffy white powder.

Peptic digestion and application to the affinity column

A 12-mg amount of performic acid-oxidized insulin (or derivative) was dispersed in 0.3 ml of pH 1.9 buffer (formic acid–acetic acid–water, 1:4:45), then 1.5 mg of pepsin (Sigma, St. Louis, MD, U.S.A.) was dissolved in 100 μl of pH 1.9 buffer and

50 μl were added to each sample. After incubating overnight at 37°C, 10-μl aliquots were subjected to high-voltage paper electrophoresis at pH 1.9 and 6.5 and the sheets cut and the separate tracks stained with cadmium–ninhydrin, Pauly, Sakaguchi and α-nitroso-β-naphthol stain. The digests were adjusted to pH 8 with pure N-ethylmorpholine and applied to the borate column as described above for undigested material. Fraction 2 (unbound material) and fractions 7 and 8 (pooled material that bound to the column and was then eluted at low pH) were freeze-dried, taken up in 200 μl of water and 2-μl aliquots were electrophoresed at pH 1.9 and 6.5. Aliquots of 2 μl were hydrolysed in the presence of mercaptoacetic acid for amino acid analysis. Aliquots of 2 μl were treated with 100 μl of 0.2 *M* hydroxylammonium chloride which had been adjusted to pH 7.0 with sodium hydroxide solution, at 37°Cvfor 23 h and were then hydrolysed in the presence of mercaptoacetic acid.

Isolation and analysis of the arginine-containing peptide from CHD-modified insulin

A mixture of 20 mg of CHD-modified insulin (C) and 1.3 mg of V8 protease (Miles Labs., Slough, Great Britain) was dissolved in 1 ml of 1 % N-ethylmorpholine, which had been adjusted to pH 8.0 with acetic acid, and applied to the borate column equilibrated with the N-ethylmorpholine buffer. A 0.2-ml volume of V8 protease solution (2 mg/ml in the same buffer) wass then applied and the flow stopped. After overnight incubation at room temperature (28°C), the column was eluted as described above. Fraction 7 (opalescent) was clarified by addition of 1 drop of glacial acetic acid. The UV spectra of fractions were determined. Unbound and bound material (fractions 2 and 7, respectively) were dried down, taken up in 300 μl of water plus 50 μl of pH 1.9 buffer and 10-μl aliquots were subjected to high-voltage paper electrophoresis at pH 1.9. Fraction 7 was then dried down before treatment with pentane-2,4-dione (reagent grade; BDH, Poole, Great Britain)[8]. Analytical electrophoresis at pH 6.5 showed evidence of approximately 50 % conversion. This was thought to be due to traces of the pH 1.9 buffer neutralizing some of the triethylamine, so the pentane-2,4-dione treatment was repeated. Analytical electrophoresis at pH 6.5 then showed that conversion was essentially complete. The peptide derivative was taken up in 500 μl of 1 % (w/v) ammonium hydrogen carbonate solution, pH 8, and incubated at 37°C for 5 h with α-chymotrypsin (Sigma), added as 50 μl of a 2 mg/ml solution in water. After removal of solvent in a vacuum desiccator, the digest was dissolved in dimethyl sulphoxide, trifluoroacetylated and permethylated; the product was taken up in chloroform and 7 % of the solution analysed by GLC–MS as previously described[6].

RESULTS AND DISCUSSION

Formation of the adduct

When porcine insulin was subjected to reaction conditions recommended for proteins[10], amino acid analysis (A, Table I) showed extensive but nevertheless incomplete derivatization of the single arginine residue at position B22. This was thought to be due to steric hindrance, so denaturing conditions were considered. Guanidinium chloride, a commonly used protein solubilizing and denaturing agent, would be expected to complete effectively with arginyl side-chains for the CHD reagent, so no experiments were performed with it. Urea, the other commonly used denaturant, is also known to react with 1,2-diketones and this was thought to preclude its use during

TABLE I

RESULTS OF AMINO ACID ANALYSIS OF PORCINE INSULIN TREATED IN VARIOUS WAYS (A–D, SEE TEXT)

MA = mercaptoacetic acid; PI = porcine insuline. The results are expressed as ratios, normalised to Leu = 6.00. "Bound" refers to material which bound to the borate column.

Amino acid	*A*		*B*		*C*		*D*		*Bound*		*PI***
	HCl	*HCl/ MA**	*HCl*	*HCl/ MA**	*HCl*	*HCl/ MA**	*HCl*	*HCl/ MA**	*HCl*	*HCl/ MA**	
Asp	3.19	3.08	2.99	3.26	2.99	3.14	3.01	3.17	3.48	3.21	(3)
Thr	2.25	2.54	2.00	2.90	1.98	2.69	1.98	2.65	1.89	1.85	(2)
Ser	3.06	2.67	2.89	2.76	2.81	2.68	2.82	2.72	2.69	2.52	(3)
Glu	7.13	7.13	7.09	7.18	7.15	7.08	7.12	7.26	6.98	6.74	(7)
Gly	3.85	3.80	3.60	4.00	3.81	4.03	3.64	3.67	3.88	3.50	(4)
Ala	2.09	2.02	1.88	2.02	1.94	1.92	2.00	2.02	1.95	1.93	(2)
Val	3.06	3.02	3.09	3.12	3.09	3.14	3.14	3.21	3.11	3.19	(4)
Cys_2	2.35		2.50		2.38		2.45				(3)
Ile	1.20	1.09	1.25	1.11	1.21	1.15	1.22	1.17	1.30	1.15	(2)
Leu	6.00	6.00	6.00	6.00	6.00	6.00	6.00	6.00	6.00	6.00	(6)
Tyr	3.29	4.01	3.87	4.03	3.22	3.95	3.16	4.08	3.01	3.92	(4)
Phe	2.88	2.87	3.02	2.87	2.89	2.81	2.87	2.87	2.85	2.89	(3)
	2.02	2.61	2.06	2.01	2.04	2.62	2.01	2.62	2.01	2.82	(2)
Lys	0.76	0.82	0.70	0.75	0.84	0.85	0.84	0.85	0.88	0.87	(1)
Arg	0.42	0.28	0.98	0.94	0.28	0.14	0.31	0.17	0.22	<0.05	(1)

* Values obtained after hydrolysis in the presence of mercaptoacetic acid, which prevents regeneration of arginine from its CHD adduct which is otherwise about 20%[10].

** Composition of porcine insulin[23]. Proline (1 residue) was not quantitated.

*** Hydrolysis of the CHD adduct of arginine in the presence of mercaptoacetic acid gives rise to a neutral product[10] which elutes on our analyser at the histidine position[7].

modifications with CHD[17]. However, guanidines are more reactive than urea[18], so one might expect to achieve a degree of arginine modification in urea solutions. This was found to be the case: a higher degree of derivatization of porcine insulin may be achieved in 4 or 8 *M* urea (Table I). The slight difference between the 4 and 8 *M* results may not be significant. Analysis of the products by cellulose acetate electrophoresis confirmed the results of amino acid analysis. In a phosphate–urea buffer (pH 7.2), CHD-modified insulin is not separated from native insulin. In a borate–urea buffer, CHD-modified insulin runs towards the anode about 1.4 times faster than unmodified insulin, consistent with the introduction of an extra negative charge on complexation with borate. The N-terminal cyanogen bromide fragment of horse-heart cytochrome-*c*, consisting of residues 1–65 and the haem prosthetic group, was also successfully modified at the single arginine residue with CHD in 4 *M* urea (Table II). The use of at least 4 *M* urea is recommended whenever quantitative reaction of CHD with the arginine residues of polypeptides or proteins is being attempted.

Isolation of CHD-modified peptides by affinity chromatography

Substances possessing 1,2- or 1,3-diol functions that can attain a suitable orientation are able to form covalent complexes with borate. Complex formation, which is reversible, is strongly favoured at alkaline pH. Borate attached to a solid support may thus be used to bind specifically such substances[19,20], which may then be released at a

TABLE II

RESULTS OF AMINO ACID ANALYSIS OF THE CHD-ADDUCT OF THE N-TERMINAL CNBr FRAGMENT OF HORSE HEART CYTOCHROME-*c*

All values normalized to Leu = 3.00.

Amino acid	*HCl/MA*	*HCl/ MA**	*PP***
Asp	5.09	5.18	(5)
Thr	6.19	5.90	(7)
Glu	6.68	6.59	(7)
Gly	10.21	9.95	(10)
Ala	3.15	2.99	(3)
Val	2.22	2.10	(3)
Ile	1.56	1.42	(2)
Leu	3.00	3.00	(3)
Tyr	0.99	0.88	(1)
Phe	2.49	2.31	(3)
His***	4.09	4.30	(3)
Lys	10.82	11.54	(11)
Arg	0.28	0	(1)

* See footnote to Table I.

** Composition of the polypeptide[27].

*** See footnote to Table I. Proline (2 residues), cysteine (2 residues) and homoserine (1 residue) not determined.

later stage. This specific recognition of a target species by an immobile ligand has become known as affinity chromatography. *m*-Aminophenylboronic acid is a suitable ligand in that it possesses a nucleophilic group for attachment to an activated support, it is a stable compound, it may be quantitated in solution by UV spectrophotometry and it is readily available. This ligand was coupled to epoxy-activated Sepharose 6B, a support found to be preferable to cyanogen bromide-activated Sepharose 4B[7]. In preliminary experiments, the column produced demonstrated an ability to bind selectively the CHD–guanido adducts of arginine and of Ser–Pro–Phe–agmatine (Agm). As borate–diol complexes are unstable at low pH, it proved possible to release the adducts with dilute acetic acid. The adduct is thus released by a volatile solvent at a pH at which it is stable even in the absence of borate.

In addition to binding small peptides carrying the diol group, the column is equally satisfactory when modified polypeptides are used. Unmodified insulin (B, see Table I) was found not to bind to the borate column (100% of the absorbance at 280 nm was found in fractions 1 and 2), whereas modified insulin (D, see Table I) does bind (15% of the absorbance at 280 nm eluted in fractions 1 and 2 and 85% in fractions 7 and 8; the latter figure agrees well with the value of 83% for the diol content of sample D deduced from the amino acid analysis result in Table I). Bound material, released by 1% acetic acid, was found to be completely derivatized (Table I).

Application to the borate column of a peptic digest of performic acid-oxidized, CHD-modified insulin led to the isolation of a set of peptides containing the modified arginine residue. The digest before application contained a set of peptides similar in

electrophoretic mobility to those which stained for arginine in the case of the control; electrophoresis showed that these were selectively bound on application to the borate column and were released, in fraction 7, on decreasing the pH of the eluent. UV spectrophotometry, electrophoresis and amino acid analysis showed that no peptides were present in fraction 7 of the control. Amino acid analysis of the isolated modified peptides was consistent with the expected cleavage pattern of pepsin around the modified arginine residue:

–Glu–Ala–Leu–Tyr–Leu–Val–Cys–Gly–Glu–Arg–Gly–Phe–Phe–Tyr–Thr–Pro–Lys–Ala
↑ ↑ ↑ ↑ ↑ ↑

The yield based on the Gly, Glu, Leu and Arg values of this analysis was 1 μmol (50%).

The CHD–diol group is stable even in the absence of borate at the low pH required for peptic digestion. Pepsin is a very convenient protease to use on this account. Digestion with trypsin may be performed in a phosphate buffer at pH 6.8 without decomposition of the CHD adduct[15]. If alkaline conditions are required for the digestion, as is sometimes the case, borate should be present in order to avoid substantial loss of the diol group[10,11]. If a high concentration of borate is used, say 0.1 *M*, then this complexes with the diol group of the adduct at the pH used for application to the borate column and effectively prevents binding to the column. For example, less than 10% of CHD-modified insulin binds when applied in 0.1 *M* sodium borate at pH 8 to a column equilibrated and then eluted with 1% N-ethylmorpholine acetate (pH 8). Although it should be possible, by using a longer or more highly substituted column, a lower borate concentration and perhaps a lower pH, to find conditions suitable for the application of alkaline digests, another possibility is to carry out the digestion with the sample bound to this borate column itself. This has been achieved. Digestion with V8 protease[21] of modified insulin bound to the borate column led to the isolation of a single peptide of electrophoretic mobility[22] at pH 6.5 of $M_{Asp} = -0.49$, and at pH 1.9 $M_{Ser} = 1.16$. The expected mobilities of Arg(DHCH)–Gly–Phe–Phe–Tyr–Thr–Pro–Lys–Ala are $M_{Asp} = -0.43$ at pH 6.5 and $M_{Ser} = 1.18$ at pH 1.9. The peptide stained red with the cadmium–ninhydrin reagent and was Sakaguchi negative.

Analysis of the modified peptide by GLC–MS

The peptide isolated by affinity chromatography from the V8 digest of modified insulin was treated with pentane-2,4-dione, digested with chymotrypsin, N-trifluoroacetylated and permethylated and an aliquot of the products analysed by GLC–MS. The selected ion record of *m/e* 150, diagnostic of the modified arginine side-chain, showed one maximum at a retention time of 28 min 22 sec. A full spectrum of the modified arginine-containing tripeptide Arg–Gly–Phe was obtained (Fig. 1), corresponding to residues 22–24 of the B-chain of porcine insulin[23].

CONCLUSION

It is possible to use a column of immobilized borate to isolate arginine-containing peptides modified with cyclohexane-1,2-dione from a proteolytic digest of a modified polypeptide carried out either at acidic or alkaline pH. The modified arginine

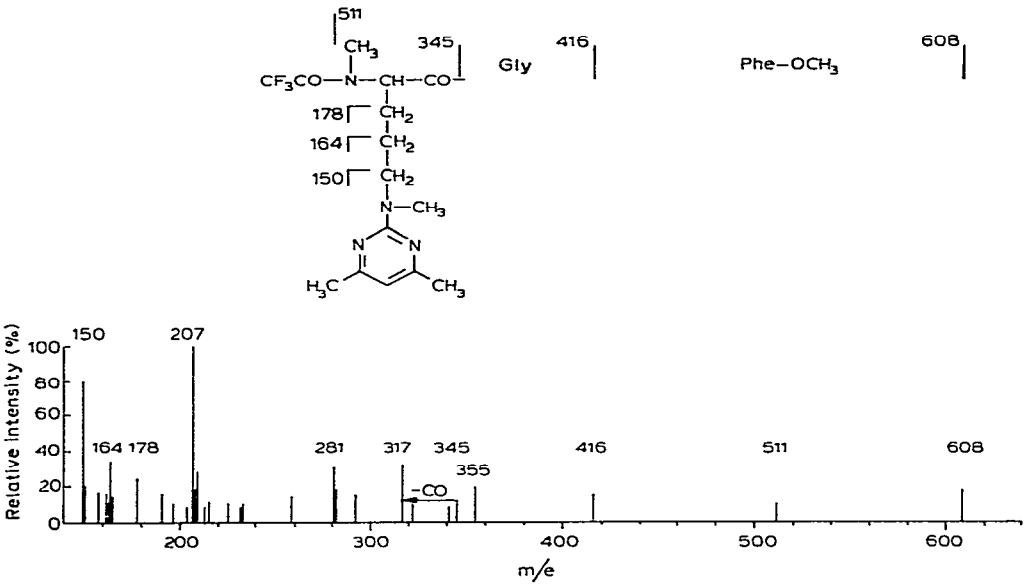

Fig. 1. Mass spectrum showing the sequence "Arg"–Gly–Phe. The arginyl residue has been converted into a residue of 4,6-dimethylpyrimidyl-2-ornithine. In this figure, the usual three-letter code is used to denote the N,O-permethylated amino acid residues. This is the raw mass spectrum ($m/e > 150$) stored as scan 185 of the GLC–MS run described in the text. Ions appearing at m/e 207, 281, 341 and 355 are due to column bleed. The sequence of the tripeptide is easily deduced via the strong sequence ions.

residue may be identified in sequence by GLC–MS. Since the completion of this work, a paper has appeared[24] describing the isolation, on a commercially available borate gel, of an arginine-containing peptide after reaction with CHD. It is of interest that the products of modification of arginine residues with butanedione in borate buffer[25] and with phenylglyoxal in borate buffer[26] are also believed to contain the 1,2-diol moiety and so could in principle be isolated by affinity chromatography on a column of immobilized borate.

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