Journal of Chromatography, 500 (1990) 697–704
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 809

FORMATION OF A CYSTEINE–ACRYLAMIDE ADDUCT IN ISOELECTRIC FOCUSING GELS

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(Received June 14th, 1989)

SUMMARY

Radical polymerization of acrylamide gels, even under carefully controlled conditions, rarely exceeds 90% conversion of the monomers into the polymer matrix. Thus, a 5%T gel will contain 30–60 m*M* unreacted free acrylamide. During isoelectric focusing (IEF), at pH > 8, free SH groups in proteins can rapidly react with the double bond of the free monomers in the gel. When Cys is incubated at room temperature with 30 m*M* acrylamide at pH 9.0, addition to the double bond is complete in 90 min. The existence of the Cys–acrylamide adduct has been ascertained by NMR, mass spectra and elemental analysis of the purified compound. It is suggested that acrylamide gels for IEF be treated with the same protocol adopted since 1980 for immobilized pH gradient gels: an "empty" matrix should be prepared, washed free of contaminants (unreacted monomers and catalysts, *e.g.*, 3 × 1 h in excess distilled water) and stored dry for subsequent use.

INTRODUCTION

In the course of our 8-year experience with immobilized pH gradients (IPG; for a review see ref. 1), we have recently become aware of oxidative phenomena occurring during the IPG run in the gel matrix. For example, during the analysis of urokinase (an alkaline protein, p*I* ≈ 10, containing 24 Cys residues) a multitude of molecules was separated, with p*I*s in the pH range 7–10, which were attributed to the existence of a number of species with SH, native –S–S– and possibly "scrambled" –S–S– groups[2].

We could subsequently demonstrate that, during gel polymerization at appropriate pH, the four alkaline buffers (the p*K* 6.2, 7.0, 8.5 and 9.3 species) were oxidized

0021-9673/90/$03.50

to different extents with formation of N-oxides[3]. These R_3–N^+O^- species are in turn oxidizing and attack free Cys residues in proteins by transforming them into –S–S– bridges. At alkaline pH values, this results in spurious bands, since the SH residue is partly or fully ionized and thus contributes to the negative surface charge. Such a phenomenon was clearly demonstrated in the case of human α-globin chains. The extent of this reaction was assessed by preparing an IPG gel buffered at a constant pH of 9.0, washing it extensively, crushing it to particulate material and incubating under controlled conditions (anaerobic and aerobic) with 10 m*M* Cys. In the system Cys + IPG gel + O_2, full oxidation to Cys_2 was attained in only 3–4 h whereas under anaerobic conditions and in an IPG gel reduced with ascorbate, full Cys protection could be ensured[4].

The next logical step was to ascertain whether such oxidative phenomena would occur also in conventional isoelectric focusing (IEF) in the presence of soluble, carrier ampholyte (CA) buffers. CAs are a multitude of oligoamino, oligocarboxylic amphoteric species, having M_r from 600 (the most basic) to 900 (the most acidic) Da[5,6]. Because CAs contain a number of primary to tertiary amino groups, it was found that during gel polymerization with persulphate, oxidation occurs for all CA ranges (acidic to basic) in essentially all commercial brands (Ampholine from LKB and Pharmalyte from Pharmacia being used as representative compounds)[7]. Oxidized CAs also were able to oxidize, during an IEF separation, Cys groups in proteins[7]. We therefore set up a model system for studying the kinetics of free Cys oxidation by incubation with oxidized CAs extracted from an IEF gel. However, we were confronted with a second phenomenon reported here for the first time in the electrophoresis literature: addition of the SH group to the double bond of unreacted acrylamide. Since, on extraction of CAs from a polyacrylamide gel, a sizable amount of unreacted monomers (30–60 m*M*) is carried along, it was found that the latter species would rapidly react with the SH group before the onset of oxidative phenomena.

EXPERIMENTAL

Materials

Cysteine, cystine, cysteic acid and spectrophotometric-grade solvents [trifluoroacetic acid (TFA), acetone, deuterium oxide] were obtained from Merck (Darmstadt, F.R.G.) and Ellman's reagent (5,5′-dithiobis-2-nitrobenzoate, DTNB) from Serva (Heidelberg, F.R.G.). The HPLC apparatus was equipped with a 655 A-12 pump, a D-2000 integrator, a Model 655 A variable-wavelength detector and an L-5000 gradient programmer (all from Merck–Hitachi). Detection of dansylated compounds was by UV absorption at 254 nm. L-Cysteinesulphenic acid monohydrate was from Aldrich (Steinheim, F.R.G.). Repel- and Bind-Silane, Gel Bond PAG, the Multiphor 2 chamber, Multitemp thermostat and the Macrodrive power supply were purchased from LKB (Bromma, Sweden) and Pharmalyte and Ampholyte buffers and the protein p*I* marker kit from Pharmacia (Uppsala, Sweden). Acrylamide, N,N′-methylenebisacrylamide (Bis), N,N,N′,N′-tetramethylethylenediamine (TEMED), ammonium persulphate and Coomassie Brilliant Blue R-250 were obtained from Bio-Rad Labs. (Richmond, CA, U.S.A.).

Thin-layer chromatography (TLC)

TLC analyses were carried out on silica gel $60F_{254}$ plates (Merck), developed for 10 min with ethanol–2.5% ammonia solution (7:3) or with chloroform–methanol–water (15:10:2). The plates were stained by spraying with ninhydrin and incubation at 90°C for 20 min.

Column chromatography

For purifying reacted Cys from CA buffers, the reaction product was passed through an analytical-reagent grade cation-exchange resin, AG 50W-X4 (Bio-Rad Labs.), equilibrated in 66 m*M* citrate buffer (pH 2.2). The sample was then eluted with 0.2 *M* sodium citrate buffer (pH 3.25). As an alternative purification step (for sample characterization by NMR, mass spectrometry and elemental analysis), the reaction product (Cys-S-propionamide, see below) was chromatographed on a silica gel 60 (200–400 mesh) column (Merck) in chloroform–methanol–water (15:10:2).

Titration of free SH groups with DTNB

A 10 m*M* solution of DTNB in 300 m*M* Tris–HCl (pH 8.0) was prepared in the dark just prior to use. For the assay, 100 μl of DTNB and 10 μl of sample solutions were added to 890 μl of the above buffer. The absorbance at 410 nm was measured after 10 min against blank tubes. A calibration graph was constructed with standards of cysteine (0–12 mmol). The molar absorption coefficient of reduced DTNB was taken as 13 600, according to Ellman[8].

Preparation of dansyl derivatives

A stock solution of dansyl chloride (100 mg in 2 ml) was prepared in spectroscopic-grade acetone. Prior to use, it was diluted 1:10 in acetone. A 350-μl volume of 0.1 *M* hydrogencarbonate buffer (pH 9.5) was mixed with 20 μl of a 2.5 m*M* sample solution (Cys and oxidation products thereof) and with 100 μl of freshly diluted stock dansyl chloride solution. After incubation at 37°C for 60 min, the reaction was stopped by adding 750 μl of 25 m*M* TFA.

Synthesis of cysteine-S-propionamide

The Cys-S-propionamide standard was prepared according to Friedman *et al.*[9], as follows: a 0.01 *M* solution of Cys (1.21 g in 20 ml of water) was titrated to pH 8.1 with dilute ammonia. To this 0.011 *M* acrylamide (0.78 g) was added and the reaction mixture stirred under nitrogen at room temperature for 48 h. The precipitate formed was filtered off and recrystallized from ethanol–water (75:25).

Incubation of Cys with oxidized CAs and unreacted acrylamide

Two IEF gels were made to contain 5%T, 4%C and 5% CAs in the pH range 6–9. They were 1.5 mm thick and 1 μl/ml of TEMED and 0.2% of persulphate were added. After polymerization for 1 h at 50°C[10], the gels were subjected to electrophoresis for 6 h at 300 V to discharge excess persulphate at the anode. The anodic and cathodic gel areas were excised and eliminated (20% of the total gel length) and the central gel portion was crushed and extracted overnight with ethanol–water (1:1) (a four-fold volume excess) in a 500-ml reaction flask equipped with a mechanical stirrer and flushed with nitrogen. After eliminating the gel particles, the ethanol was evap-

orated and the CA–acrylamide solution was rendered 200 m*M* in borate buffer (pH 9.0). To this solution Cys was then added to a final concentration of 10 m*M*. The reaction was followed by collecting and analysing aliquots at 30-min intervals by TLC and with Ellman's reagent. In parallel, the same stock Cys solution was incubated in another flask in the absence of IEF gel extracts and under a nitrogen atmosphere, as above.

High-performance liquid chromatographic (HPLC) analysis

The samples incubated in CAs and purified by ion-exchange chromatography, after dansyl derivatization, were injected into the column (300 × 4 mm I.D. microparticle reversed-phase Hypersil C_{18} from Gyncotech) of an HPLC apparatus. Gradient elution (flow-rate 10 ml/min) was performed with two solutions: (A) acetonitrile–25 m*M* sodium trifluoroacetate (TFA) buffer of pH 7.2 (10:90) and (B) TFA buffer of pH 7.2–acetonitrile (30:70). The gradient had the following composition at the times indicated (t, min): (t_0–t_4, 96% A, 4% B; t_{12}, 70% A, 30% B; t_{16} and t_{19}, 30% A and 70% B; t_{21}, 96% A and 4% B. The column and sample temperature was 40°C. Calibration graphs were constructed with standards (2.5 mmol) of dansylcysteine, cysteic acid, cystine and cysteinesulphenic acid injected into the HPLC column.

NMR and mass spectra

Proton nuclear magnetic resonance analysis was carried out with a Model AM-500 500-MHz NMR spectrometer (Bruker, Rheinstetten, F.R.G.) for solutions in deuterium oxide, using 3-(trimethylsilyl)tetradeuterosodium propionate (TSP) (Wildman, Buena, U.S.A.) as internal standard. Mass spectra were measured with an LKB 2091 gas chromatograph–mass spectrometer. The analysis was done by direct injection of the sample.

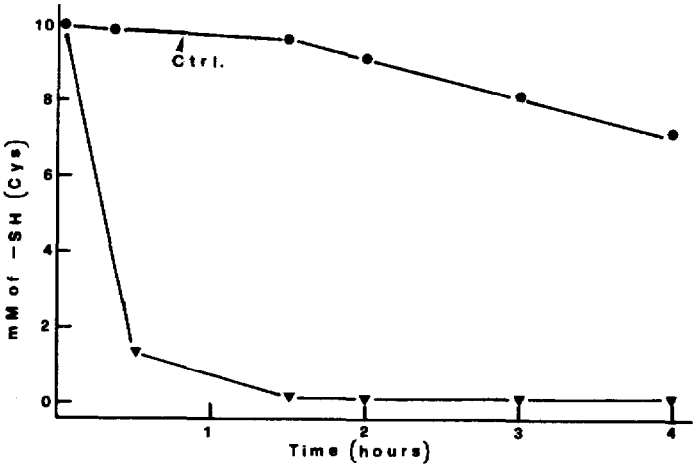

Fig. 1. Kinetics of free SH disappearance in Cys on incubation with IEF gel extracts. 10 m*M* Cys was incubated with an extract from a pH 6–9 IEF gel in the presence of 200 m*M* borate buffer (pH 9.0). The gel had been pre-focused for 6 h at 300 V to discharge persulphate at the anode. At the given time points, duplicate aliquots were taken and assayed for free SH by the Ellman reaction. Crtl.: control, incubated aerobically in the absence of the gel extract.

RESULTS

When 10 m*M* Cys is incubated with 1% oxidized carrier ampholytes extracted from a gel after a pre-run (see Experimental), free SH groups, as monitored by the Ellman reaction, are seen to disappear completely in a 90-min incubation time (Fig. 1). By following the reaction progress by TLC, a new compound, with an R_F different from that of Cys, is observed (not shown). In order to establish its identity, the reaction product was purified from contaminant CAs by cation-exchange chromatography and subsequently dansylated. On analysis by HPLC, a peak with a retention time of 15.13 min was eluted (see Fig. 2; the unknown is labelled with a question mark). As in previous analyses we had characterized a number of Cys derivatives (see Fig. 2 in ref. 7), we could exclude the formation of Cys, Cys_2, cysteic acid, cysteinesulphenic acid and S-cyanoethyl-Cys.

We therefore resorted to NMR analysis of the above product (purified on a

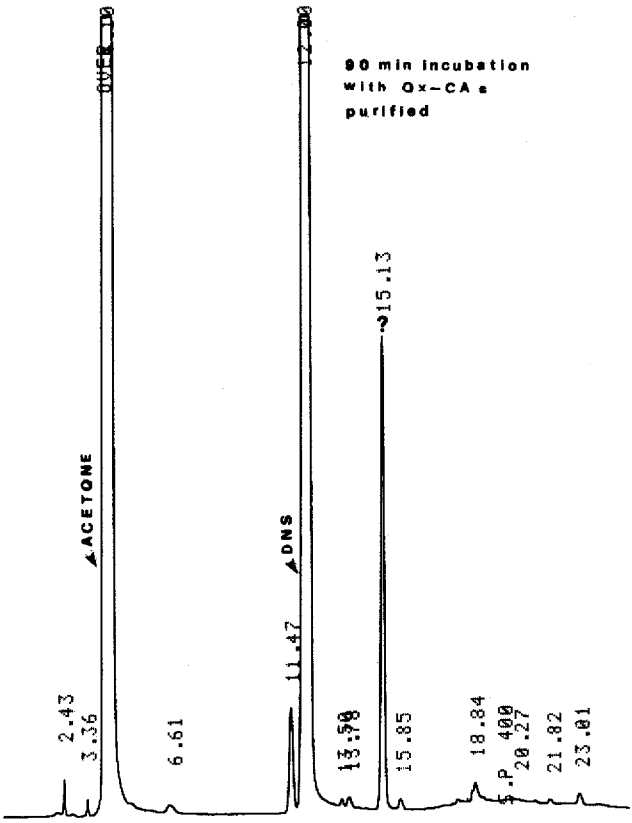

Fig. 2. HPLC of the Cys incubate in Fig. 1. After complete disappearance of free SH groups (4-h point), the incubate in Fig. 1 was purified from carrier ampholytes on a cation-exchange column, dansylated and analysed by HPLC. The peak marked with a question mark was found not to be Cys, Cys_2, cysteic acid, cysteinesulphenic acid or S-cyanoethyl-Cys. Its identity (cysteine-S-propionamide) was ascertained by NMR. Numbers indicate retention time in min.

silica gel column) to investigate its structure. Such spectra could only be interpreted by hypothesizing a Cys–acrylamide adduct. The above compound was therefore synthesized, crystallized and subjected to NMR. The identity of the two spectra confirmed the presence of cysteine-S-propionamide, via the following signals (chemical shifts, δ, being in ppm and coupling constants, J, in Hz): cysteine moiety: CH, δ = 3.94 (four lines, vicinal J = 4.3 and 7.5), 1H; CH_2, two pseudo-quartets, centred at δ = 3.18 (geminal J = −15 and vicinal J = 4.3), 1H and δ = 3.065 (geminal J = −15 and vicinal J = 7.5), 1H; propionamide moiety: CH_2CO, δ = 2.87 (triplet, vicinal J = 7.5), 2H; CH_2S, δ = 2.62 (triplet, vicinal J = 7.5), 2H.

Mass spectra of the two compounds (the synthetic product and Cys incubated with an IEF gel extract) were analogous and gave fragments of m/z = 193 ($M^+ + 1$), 176 ($M^+ - 16$), 147 ($M^+ - 45$) and 104 ($M^+ - 88$). The identity between the Cys derivative obtained by incubation with an IEF gel extract and Cys-S-propionamide was also confirmed by elemental analysis of the products purified by column chromatography and crystallized from methanol: calculated for $C_6H_{12}N_2O_3S$ (M_r = 192), C 37.51, H 6.25, N 14.58, S 16.66; found C 37.56, H 6.31, N 14.62, S 16.61%. The formula of the Cys–acrylamide adduct is thus $H_2NCH(COOH)CH_2SCH_2CONH_2$.

DISCUSSION

While searching for potential oxidation artefacts of SH groups by oxidized carrier ampholytes in an IEF run, we stumbled upon a well known reaction in organic chemistry, *i.e.*, addition of thiols to an acrylic double bond, previously unreported in the electrophoresis literature. The reason for this lies in the fact that, in the preparation of the gel matrix, the conversion of monomers into the polymer rarely exceeds a 90% yield[10]. Thus, in a 5%T matrix (as routinely used in IEF), the amount of unreacted acrylamide could be as high as 30–60 mM. This is a vast excess compared with potentially reacting SH groups of proteins undergoing IEF analysis (present at a level of the order of nanomoles to micromoles per litre). As IEF fractionations occur along a pH gradient, it is clear that, at appropriate pH (in general at pH > 8), free SH groups can add to the double bond of unreacted acrylamide. This is a well known reaction[9], and in fact we had used the Friedman *et al.*[9] approach to protect free SH groups with acrylonitrile during a dansylation step[4].

Curiously, however, there seems to be little concern about this potential artefact in the electrophoresis literature. The only study that we could find on similar phenomena is a report by Dirksen and Chrambach[11] on the reaction of free acrylamide with the NH_2 group of Gly. According to these workers, when 3.12 mM Gly is incubated with 70 mM acrylamide at pH 9.05 and 22–25°C in Tris (6.3 g/l), complete disappearance of free NH_2 groups is seen after *ca.* 600 h (*i.e.*, 25 days). Only when 3.12 mM Gly is incubated with 1500 mM acrylamide does the α-amino group disappears in 24 h at pH 9.05 and in 30 min at pH 11. The same workers[11] further hypothesized that "in presence of urea, persulphate, riboflavin, TEMED and acrylamide monomer" there could be an environment "providing a free radical donor species that can react with proteins, possibly at the tyrosyl groups". This is the only report that hypothesized two possible reactions of excess, unreacted acrylamide with protein groups, namely (a) free amino groups and (b) the OH-group of Tyr. We should like, however, to emphasize the following points: (i) these two reactions have

only been hypothesized, and no direct chemical evidence has been given, *e.g.*, in terms of synthesizing the hypothetical derivatives, crystallizing them and determining their structure, as reported here; (ii) given the p*K* values of the reacting groups (α-amino = 9.6 and Tyr-OH = 10.2), it is highly unlikely that these addition reactions could indeed occur during an IEF experiment. Considering the above conditions (600 h at 25°C, pH 9.05 in 70 m*M* acrylamide), there is virtually no chance that in a typical IEF run (2°C, *ca.* 50 m*M* acrylamide, maximum 3 h focusing time, allowing for similar pH values) double-bond addition to either α-amino or Tyr-OH would occur. On the contrary, as demonstrated here, owing to its lower p*K* value (8.3), the free SH group is the only one that has a chance of undergoing a fast reaction with free acrylamide.

Are our previously published data on Cys oxidation then wrong[3,4]? We think not. Since their inception in 1980, IPG gels have been subjected to a rigorous polymerization routine: after gelling, the cassette is opened and the gel subjected to a thorough washing cycle (typically 3 × 1 h in excess distilled water, *e.g.*, 500 ml for a 16-ml gel volume, precast gels from the manufacturer being washed up to ten times). With such drastic washing, we have demonstrated that there are no acrylamide leachables present down to the picomole level[12].

Given the above findings, it is clear that even in conventional CA IEF gels the stringent procedure used for IPG gels should be adopted; we strongly recommend (and manufacturerers should market them) that "empty" gel matrices should be made, washed, dried onto their plastic Gel Bond foil and stored frozen, ready to be reswollen in any additive. This will be like having a disposable fractionation column, something that can be done inexpensively in electrophoresis, but which is not easily available in chromatography. This will automatically overcome two problems: (a) prevent oxidation of CAs by persulphate during the polymerization; and (b) eliminate noxious free monomers (and ungrafted oligomers).

Up to the present, CA IEF and IPGs have been something of an "art"; from now on, they should be more science than art.

ACKNOWLEDGEMENTS

This work was supported in part by a grant from Progetto Finalizzato Chimica Fine II, CNR, Rome. We thank Dr. M. Corti (University of Milan) for the HPLC analyses.

NOTE ADDED IN PROOF

After this paper had been accepted, we became aware of another paper describing the reaction of acrylamide at alkaline pH with buffer components and protein[13]. Their reaction conditions were fairly drastic, *e.g.*, 20 m*M* amine solutions (titrated to their p*K* values) incubated in 10% T acrylamide–Bis solutions. Thus, on average, there are 1.5 *M* double bonds *vs.* 20 m*M* potentially reactive amines. For the few buffers that do react (aminoethane, taurine, glycine, morpholine), it takes 24 h to 7 days to achieve extensive adduct formation (as judged by p*K* changes) and this in a vast excess of double bonds, typically a 70–80-fold molar excess. Although the paper cited fully confirmed well known findings of addition of double bonds to primary and secondary amino groups, curiously it did not report any potential reaction between

free SH and double bonds which, according to the present findings, should be by far the most facile reaction to occur in proteins.

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