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KINETICS OF CYSTEINE OXIDATION IN IMMOBILIZED pH GRADIENT GELS

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SUMMARY

It was recently found that, during the gel polymerization step, persulphate could oxidize the alkaline Immobiline species (acryloyl weak bases used for producing immobilized pH gradients, IPG) by forming N-oxides. When focusing proteins in alkaline pH ranges, free SH groups were oxidized to –S–S– bonds, generating artefactual, higher p*I* bands. The kinetics of this phenomenon were investigated in a model system consisting in incubating free Cys with a crushed IPG gel auto-buffered at pH 9.0 under aerobic or anaerobic conditions. After appropriate incubation periods, any excess free SH was blocked with acrylonitrile. After dansylation, Cys and its oxidized derivatives were separated and quantified by high-performance liquid chromatography in a C_{18} column. Under anaerobic conditions, control Cys (incubated in the absence of IPG gel) showed 30% oxidation after a period of 12 h, whereas Cys incubated with a pH 9.0 crushed IPG gel was 100% oxidized to cystine. When the same experiment was repeated under aerobic conditions, the oxidation process was accelerated by a factor of 3–4. Successful focusing of alkaline proteins can still be achieved if (a) the IPG gel is washed in 100 m*M* ascorbic acid, prior to the run, for elimination of N-oxides from the matrix, (b) the IPG gel is run anaerobically and (c) the focusing time is kept to a minimum (*e.g.*, 4 h *vs.* the standard overnight run).

INTRODUCTION

Recently, when focusing recombinant or urine-purified urokinase [a 50 000-dalton glycoprotein consisting of two chains of 20 000 and 30 000 dalton, connected by a disulphide bridge, containing an unusually high level of Cys residues (a total of 24)], used in human therapy as a fibrinolytic agent, we detected an extreme charge heterogeneity (at least ten major and ten minor bands in the pH range 7–10)[1]. This

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protein, extensively purified by immunoaffinity and ion-exchange chromatography[2], gave in sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) a single, size-homogeneous zone and was classified as a therapeutic-grade product, already distributed by Lepetit (Gerenzano, Italy) for human use as a thrombolytic agent in acute myocardial infarction. The finding of this extensive heterogeneity was therefore a cause of great concern. In an extensive investigation[1], we could eliminate glycosylation, isoelectric focusing (IEF) artefacts [*e.g.*, binding to carrier ampholytes, (CA)] or carbamylation as potential sources of this polydispersity. Finally, a great part of this heterogeneity could be traced back to the existence of a multitude of protein molecules containing Cys residues at different oxidation levels (SH, –S–S–, possibly even cysteic acid). The cause of these different redox states of Cys in urokinase was not apparent, although part of it might be attributed to spontaneous oxidation by atmospheric oxygen, owing to the relatively high p*I* of native urokinase (*ca.* 9.5–9.8 by conventional IEF).

We subsequently discovered that, to a large extent, this oxidation power was inherent to the Immobiline matrix because, during the polymerization step, at appropriate pH values, the persulphate could oxidize, to different extents, all four alkaline Immobilines (the p*K* 6.2, 7.0, 8.5 and 9.3 species) used as buffers for focusing in basic pH regions[3]. It was hypothesized that, on exposure to ammonium persulphate, there would be addition of oxygen to the tertiary amino group of deprotonated alkaline Immobilines, producing amine oxides (R_3–N^+O^-). This reaction is facile and usually occurs readily at room temperature in water, alcohol or benzene solvents in the presence of even dilute solutions of organic peracids[4]. Hence the assumption that, on washing, or electrophoretically pre-running an immobilized pH gradient (IPG) matrix, the latter would be devoid of any oxidizing power, as harmful persulphate would be washed away or be discharged at the anode, proved to be fallacious because these "oxidizing units" were indeed anchored to the gel matrix in the form of N-oxides ($R_3N^+O^-$). As the redox potential in reactions involving nitrogens is of the order of $+0.5$ V, whereas that of similar reactions involving sulphur is of the order of -0.5 V, it was apparent that the N-oxides anchored to the IPG matrix would act as oxidizing agents on free SH groups (possibly even on –S–S– bridges) of proteins migrating through the gel, producing species of higher oxidation level. Hence the alkaline Immobiline themselves (oxidized by persulphate during polymerization) would act as electron acceptors in this redox process. During the IPG run, two reactive Cys residues in a protein would release two protons and two electrons which would be captured by an oxygen atom bound to the tertiary amine, thus reducing the latter and forming a water molecule. This phenomenon was verified when focusing human α-globin chains[3] and its suppression could be achieved by simply reducing the matrix, *e.g.*, by a washing step in 100 m*M* ascorbic acid at pH 4.5.

More recently, the same phenomenon could be observed even in conventional IEF, in presence of CA buffers[5]; when polymerizing the gel at low ammonium persulphate (AP) levels (0.025%), the α_{ox}/α_{red} ratio was only 15:85; at the normal AP levels (0.04%) this ratio was increased to 35:65, whereas at high AP levels (*e.g.*, 0.2%) the oxidized α-chain was clearly predominant (α_{ox}/α_{red} = 80:20). Here also a remedy could be found by polymerizing an "empty" gel, washing, drying it and reswelling in the presence of the desired CA interval.

In this work, we have devised an experimental model for studying the kinetics

of this oxidation process: free Cys is incubated, at pH 9.0, in presence of a crushed IPG gel and its reaction products are analysed and quantified, after blocking of free SH groups with acrylonitrile and dansylation, by high-performance liquid chromatography (HPLC) on a C_{18} column. The process was studied both under anaerobic and aerobic conditions and guidelines are given for the successful fractionation of alkaline proteins.

EXPERIMENTAL

Cysteine, cystine, cysteic acid, acrylonitrile and spectrophotometric-grade solvents [trifluoroacetic acid (TFA) and acetone] 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 Model 655 A-12 pump, a Model D-2000 integrator, a Model 655A variable-wavelength detector and a Model L-5000 gradient programmer (all from Merck–Hitachi). Detection of dansylated compounds was by UV absorption at 254 nm.

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). The plates were stained either with iodine vapours or by spraying with ninhydrin and incubating at 90°C for 20 min.

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 are added to 890 μl of the above buffer. The absorbance at 410 nm is measured after 10 min against blank tubes. The calibration graph is constructed using standards of cysteine (0–12 mmol). The molar absorption coefficient of reduced DTNB is taken as 13 600, according to Ellman[6].

Preparation of dansyl derivatives

A stock solution (100 mg in 2 ml) of dansyl chloride is prepared in spectroscopic-grade acetone. Prior to use, it is diluted 1:10 in acetone. Mix 350 μl of 0.1 *M* hydrogencarbonate buffer (pH 9.5) 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. Incubate at 37°C for 60 min; the reaction is stopped by adding 750 μl of 25 m*M* TFA.

Synthesis of S-cyanoethylcysteine

The S-cyanoethyl Cys standard is prepared according to Friedman *et al.*[7] as follows. A solution of 6.7 g (0.056 mole) of Cys in 15 ml of water is titrated to pH 8.1 with dilute ammonia. The solution is diluted to 60 ml, transferred into a 100-ml reaction flask and stirred with nitrogen flushing. A 0.057-mol (3.7-ml) amount of acrylonitrile is added and the reaction is continued with stirring for 4 h. The solution is evaporated to dryness under vacuum at 30–33°C and the residue is recrystallized first from 75% ethanol and then from 80% ethanol. At 5°C, white crystals are formed (80% yield). The IR spectrum of the product shows a nitrile signal at 2240 cm^{-1}. ^1H NMR spectrum (in deuterated TFA): δ 4.7 ppm [1H,C*H*(NH_3)COOH]; 3.53 ppm (2H, CH_2CN); 3 ppm [4H, $(CH)_2S$].

Incubation of Cys with an IPG granulated matrix

The IPG gel contains 10%T, 8%C[a] and 10 m*M* p*K* 6.2, 10 m*M* p*K* 7.0, 5 m*M* p*K* 8.5 and 5 m*M* p*K* 9.3 Immobilines titrated to a single pH value of 9.0 with the p*K* 3.6 species. After polymerization[8] and standard washing procedures[9], the gel is crushed through a 100 mesh/in.^{2} sieve and placed (a total of 5.5 g wet weight of gel) in a 100-ml reaction flask equipped with a mechanical stirrer and flushed with nitrogen. A 10 m*M* Cys solution is prepared in 100 m*M* borate buffer (pH 9.0), 10 ml of this solution are added to the flask containing the crushed IPG gel and the reaction is followed by harvesting and analysing aliquots after 4 and 12 h of incubation. In parallel, the same stock Cys solution is incubated in another flask in the absence of IPG matrix and under a nitrogen atmosphere, as above. At the appropriate time intervals, (a) duplicate aliquots are taken for immediate titration of free SH groups with Ellman's reagent, and (b) additional aliquots are harvested and acrylonitrile (10 μl of a 0.15 *m* solution, pH 8.1) is added in order to block unreacted free SH groups (this will prevent further reaction during the subsequent manipulation steps prior to HPLC analyses). After completion of reaction the pH is increased to 11.5 by addition of 6 *M* sodium hydroxide solution (this solubilizes cystine, which tends to precipitate at lower pH) and finally the reaction products are dansylated for HPLC analysis.

HPLC analysis

After acrylonitrile and dansyl derivatization, the samples, freed from gel particles, are injected into the column (300 × 4 mm I.D. microparticle reverse-phase Hypersil C_{18} from Gynkotek, München, F.R.G.) of an HPLC apparatus. Gradient elution (flow-rate 1.0 ml/min) is performed with two solutions, (A) acetonitrile–25 m*M* sodium trifluoroacetate buffer, pH 7.2 (10:90) and (B) sodium trifluoroacetate buffer, pH 7.2–acetonitrile (30:70), with the following gradient composition at the times indicated: 0–4 min, 96% A–4% B; 12 min, 70% A–30% B; 16 and 19 min, 30% A–70% B; 21 min 96% A–4% B. The column and sample temperature was 40°C. For calculating the percentage oxidation with time, calibration graphs were constructed with standards (2.5 mmol) of S-cyanoethyldansylcysteine and of dansylcystine injected into the HPLC column.

RESULTS

When standards of Cys are incubated in the presence of IPG particles containing all the four alkaline Immobilines, titrated to a constant pH of 9.0 (the approximate p*I* value of α-globin chains in an IPG gel) and analysed by TLC, it is clearly seen that there is substantial conversion to at least the oxidized cystine stage (possibly also to other oxidation products, as an additional spot is clearly visible at 5 and 10 h just below the Cys zone; see Fig. 1A). After 10 h of incubation, it is seen that no free Cys remains, most of it being converted to cystine (Cys_2) and to the unknown product below the Cys band. Conversely, when the same experiment is repeated in the presence of IPG particles which had been reduced in 100 m*M* ascorbate (pH 4.5), prior to the incubation, it is seen (Fig. 1B) that substantial amounts of Cys remain even after 10 h of incubation (in both instances the experiments were performed in atmospheric

[a] C = g Bis/%T; T = (g acrylamide + g Bis)/100 ml solution.

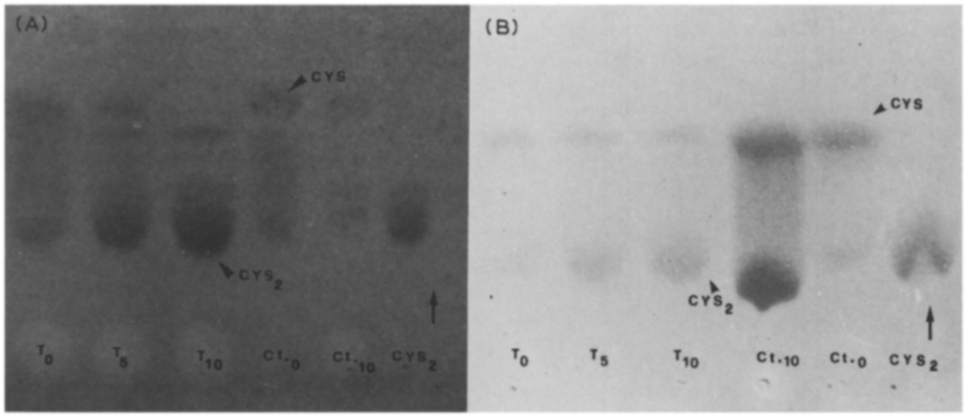


Fig. 1. Kinetics of Cys oxidation in the presence of IPG particles as monitored by TLC. 10 m*M* Cys solutions were incubated with (A) unreduced and (B) reduced IPG gel particles (pH 9.0) under aerobic conditions for 0, 5 or 10 h (T_0, T_5 and T_{10}, respectively). Ct. = Control incubations, in the absence of IPG particles; Cys_2 = standard of cystine. The vertical arrow indicates the direction of migration. The TLC plate was developed with a ninhydrin spray.

oxygen). However, it was found to be impossible to obtain reliable kinetic data by spot integration and densitometry. This system was therefore abandoned in favour of HPLC fractionation and analysis.

In HPLC, although analysis of Cys has already been reported[10], we still found the system to suffer from many practical problems. First, the reproducibility was very poor, and this was related to the time lag between the end of the reaction (incubation in the presence or absence of IPG particles) and the injection time. During the dansylation step, in fact, additional oxidation by atmospheric oxygen could take place. This problem was solved by reacting, at the end of the incubation period and prior to HPLC analysis, the excess free SH group with acrylonitrile, thus producing a stable S-cyanoethyl-Cys. In addition, it was found that the peaks of cystine were highly irreproducibile, and often absent, even though we could follow the disappearance of the Cys peak in the chromatogram and the decrement of free SH groups by the Ellman reaction. This loss of cystine was traced back to its insolubility at the pH preavailing during the incubation step with IPG particles (pH 9.0). Hence the system had to be extracted at pH 11.5. When all these precautions were taken, highly reproducible results could be obtained.

The HPLC gradient utilized was selected for its ability to separate the most common conversion products of Cys, *i.e.*, cystine and the fully oxidized cysteic acid, as we suspected that the terminal product of oxidation in presence of an IPG gel could be this latter compound. Fig. 2 shows than the HPLC method adopted can indeed separate Cys, Cys_2 and cysteic acid, in addition to the blocked derivative, S-cyanoethyl-Cys. Fig. 3A demonstrates the oxidation progress of Cys to –S–S– bonds in a control incubation during a 12-h period in the absence of IPG particles and under anaerobic conditions. It is seen that, even in this controlled environment, auto-oxidation (presumably due to traces of oxygen still present) takes place up to a level of 29% at the end of the 12-h period. However, when the same experiment is

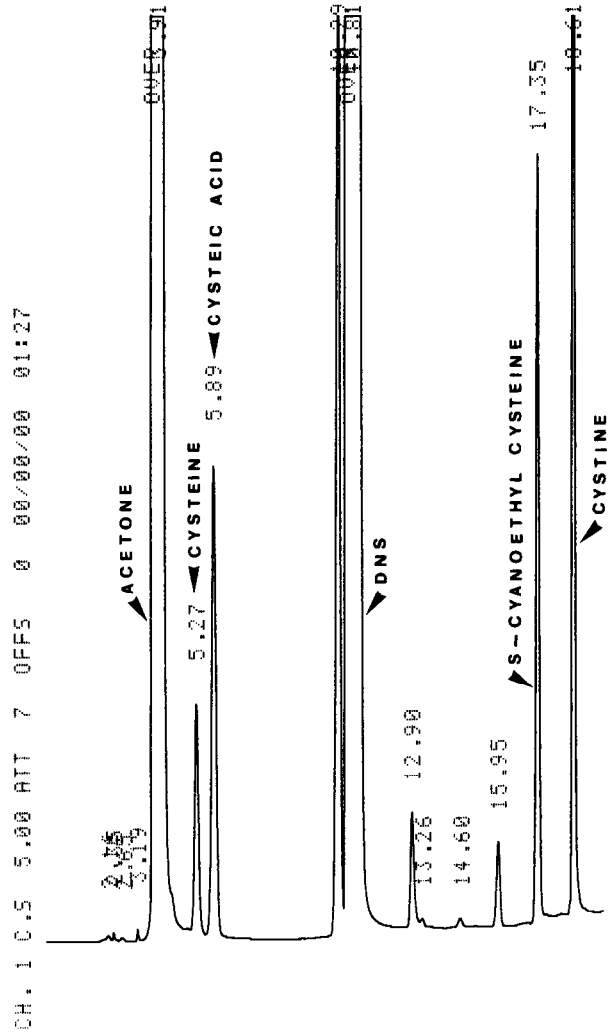


Fig. 2. HPLC fractionation of Cys derivatives. The gradient utilized (see Experimental) allows the separation of cysteic acid, cysteine, cystine and S-cyanoethylcysteine [the two other major peaks are acetone and excess dansyl (DNS)].

repeated in the presence of the pH 9.0 IPG particles (containing all four alkaline Immobiline species; Fig. 3B), at the end of this 12-h period 100% oxidation has taken place and the Cys peak has completely disappeared (in agreement with Fig. 1A).

When the two experiments are repeated under aerobic conditions, the conversion rate is accelerated by a factor of 3–4. As seen in Fig. 4A, in the control 30% oxidation has already taken place in only 4 h, whereas in the presence of IPG particles 100% oxidation has already occurred in the same time period (Fig. 4B; *cf.*, only 18% under anaerobic conditions, Fig. 3B). The kinetics of both phenomena are summarized in Fig. 5. The left-hand side shows the oxidation kinetics (under aerobic and anaerobic conditions) of control incubates and in presence of unreduced IPG parti-

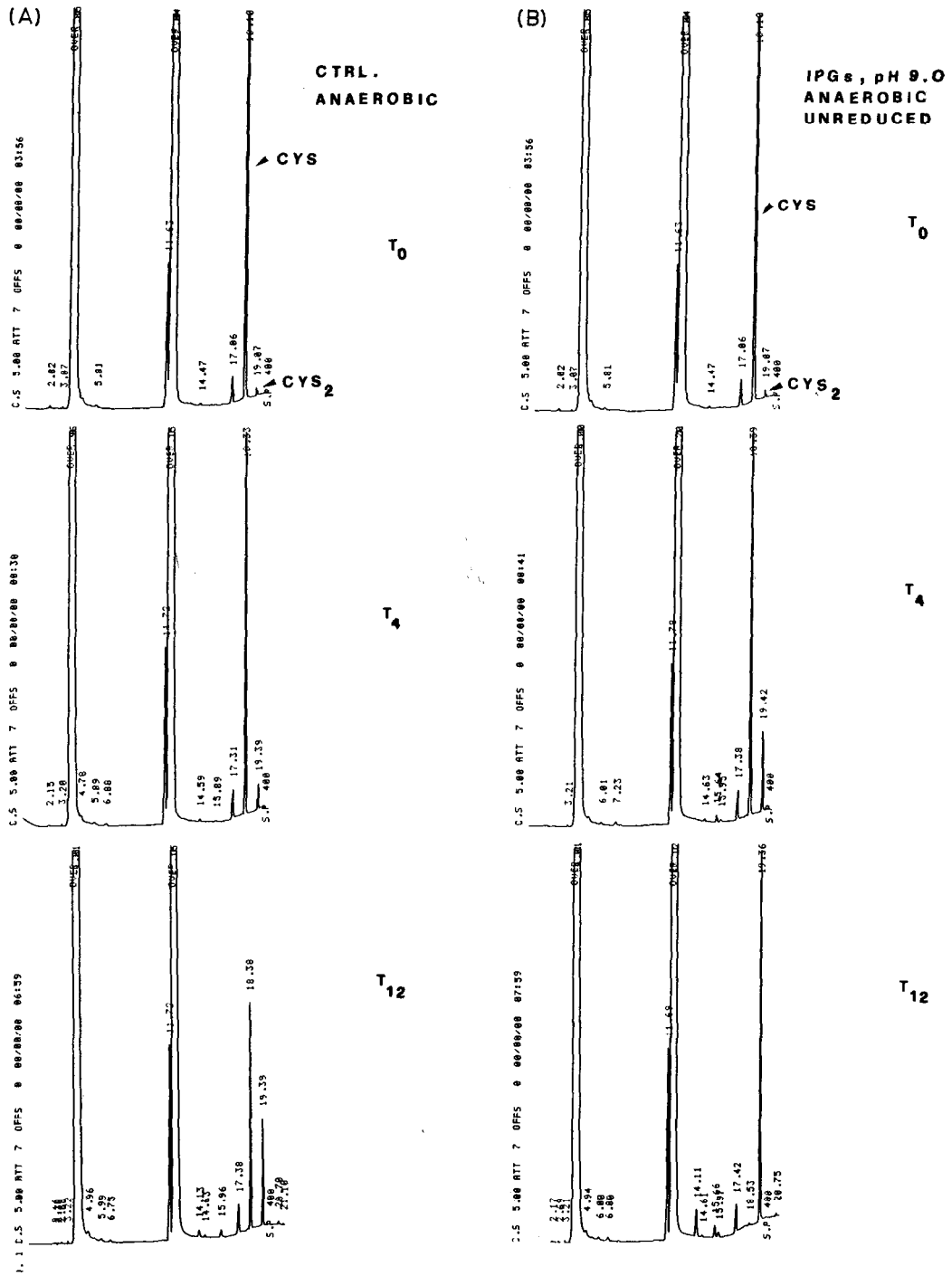
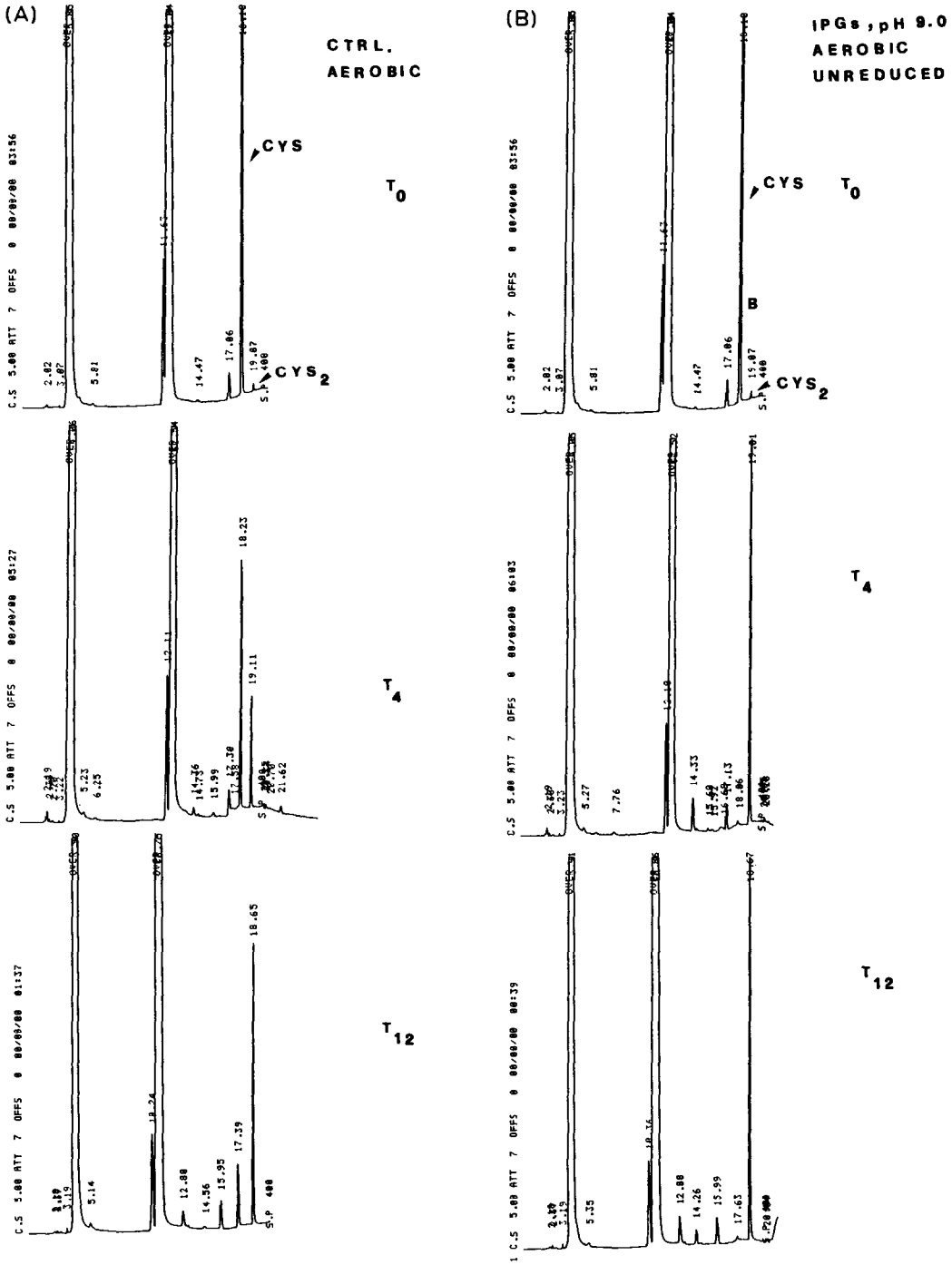


Fig. 3. Kinetics of Cys oxidation as followed by HPLC analysis. (A) Control incubations in the absence of IPG particles at 0, 4 and 12 h (T_0, T_4 and T_{12}, respectively); (B) incubations in the presence of unreduced IPG gel particles titrated to pH 9.0 at the same time intervals. In all instances the incubations were done under a nitrogen atmosphere. Cys = Cysteine; Cys_2 = cystine. Note that, in the presence of the IPG matrix, an unknown oxidation peak with a retention time of 14.11–14.47 min appears, the chemical nature of which is unknown.



F ig. 4. Kinetics of Cys oxidation as followed by HPLC analysis. (A) Control incubations in the absence of IPG particles at 0, 4 and 12 h (T_0, T_4 and T_{12}, respectively); (B) incubations in the presence of unreduced IPG gel particles titrated to pH 9.0 at the same time intervals. In all instances the incubations were done under aerobic conditions. Cys = Cysteine; Cys_2 = cystine. Note that, in presence of the IPG matrix, an unknown oxidation peak with a retention time of 14.11–14.47 min appears, the chemical nature of which is unknown. Note also the much accellerated oxidation kinetics in presence of atmospheric oxygen.

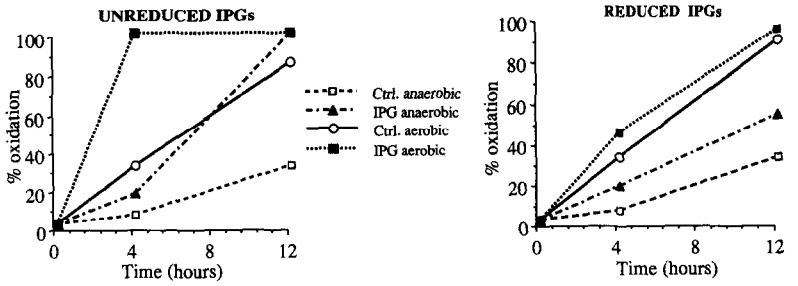


Fig. 5. Kinetics of Cys oxidation as followed by HPLC analysis. The data are presented as percentage cysteine oxidation as measured by peak integration in the HPLC traces in Figs. 3 and 4. The values reported refer to both aerobic and anaerobic conditions. Left, unreduced IPG particles; right, reduced IPG matrix. □ = Control, anaerobic; ▲ = IPG, anaerobic; ○ = control, aerobic; ■ = IPG, aerobic.

cles. On the right, the same values are reported but in the presence of a reduced IPG matrix (*i.e.*, treated with 100 m*M* ascorbate, pH 4.5). It is seen that, under anaerobic and reducing conditions, oxidation of Cys is greatly reduced. Fig. 6 gives the same data obtained by monitoring in parallel the incubates with the DTNB reaction (Ellman's reagent); even though, in this last instance, only the disappearance of free SH groups could be quantified, the two sets of data (HPLC fractionation and direct chemical analysis) agree fairly well.

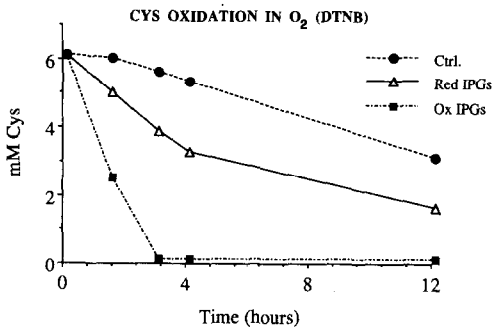


Fig. 6. Kinetics of Cys oxidation as followed with Ellman's reagent. A 6 m*M* Cys solution was incubated at pH 9.0 either as such (●, control) or in the presence of (■) unreduced or (△) reduced IPG particles. All data points refer to aerobic conditions. The disappearance of free SH groups is followed by the DTNB reaction.

DISCUSSION

A vast literature exists on the oxidation of thiols in aliphatic and aromatic compounds (for a review, see Capozzi and Modena[11]). Whereas in protein chemistry courses, we are used to dealing with only three oxidation states (free thiols, disulphides and, in extreme cases, cysteic acid), in reality thiols are oxidized by a variety of reagents to a whole series of higher oxidation products, depending on the specific reaction conditions (see Fig. 7). There are therefore two main oxidation chains, with a number of interconversions, which could occur via the hydrolytic products shown on the right-hand side of Fig. 7. In principle, all these intermediate reaction products

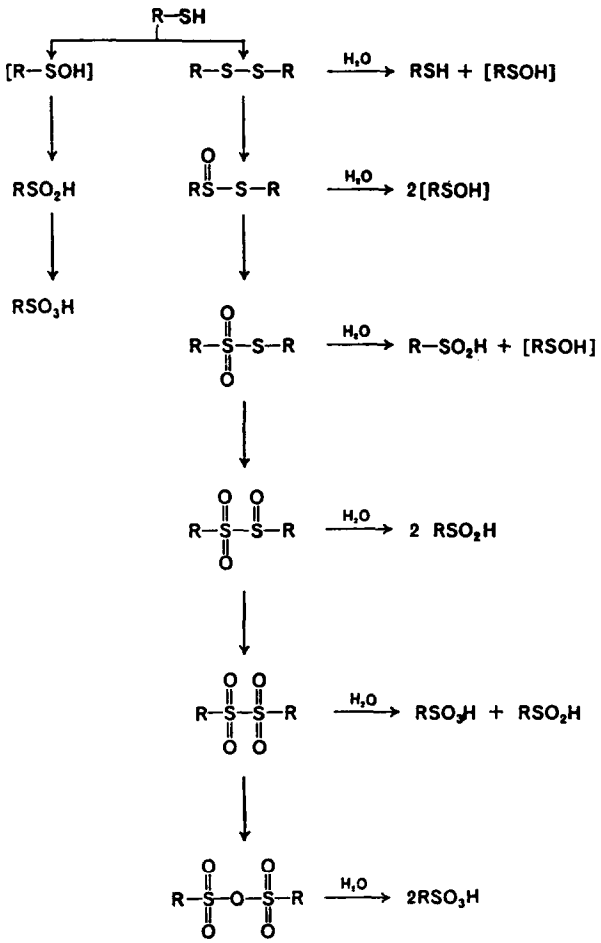


Fig. 7. Scheme of possible oxidation pathways of Cys. Note that several sulphone intermediate products of Cys_2 exist as stable compounds, in addition to the well known Cys_2 and cysteic acid terminal products. (From ref. 11.)

could exist in a thiol compound, except for the very labile sulphenic acid (shown in brackets), the isolation of which is virtually impossible. Perhaps the most popular oxidation pathway for thiols is via molecular oxygen. The ease of oxidation of thiols on exposure to air is well known, as is the sensitivity of this reaction to catalysts, such as metal ions, UV light and other initiators of radical reactions[12]. It is also reported that auto-oxidation of thiols is accelerated by bases. Of great concern to protein chemists dealing with electrophoresis in polyacrylamide gels is the chemical oxidation of thiols by peroxidic compounds. The oxidation of thiols by hydrogen peroxide, alkyl hydroperoxides and peroxy acids is a well known reaction[13]: the initially formed product is the corresponding disulphide in most instances, which can easily be further oxidized by excess of oxidant. As polyacrylamide gels are routinely prepared by radical reactions in the presence of persulphate ions, it is in general recommended to deplete this strong oxidant electrophoretically by discharging it at the anode during a

short prerun. With this precaution, electrophoresis of native proteins has always been considered to be a safe procedure, as no residual oxidizing activity is left in the gel matrix after passage of the persulphate boundary (*i.e.*, the pendant amido groups are not oxidized by persulphate).

With our recent findings, it became clear that in IPG gels, all the alkaline Immobilines (except for the quaternary titrant, QAE-arylamide) can be oxidized, at appropriate pH, to N-oxides[3] of the type $R_3N^+–O^-$, and that in CA–IEF gels, the carrier ampholyte buffers are oxidized forming primary to tertiary amine oxides[5]. Hence in an IEF experiment, the idea of prerunning the gel to discharge harmful persulphate has proved to be fallacious, as neither the CA buffers nor the Immobilines can leave the gel. The former will migrate at different velocities to their p*I* position until a steady state is reached; the latter are anchored to the gel matrix and therefore can only oscillate about their bond in a strong electric field. We have contended that these N-oxides could act as "oxidizing units" on thiol groups of proteins according to the following scheme:

$$2Pr–SH + R_3N^+–O^- \rightarrow Pr–S–S–Pr + R_3–N + H_2O$$

where Pr–SH represents a protein macroion with a reactive thiol and $R_3–N^+–O^-$ is an oxidized alkaline Immobiline (see Chiari *et al.*[14,15] for their formulae and properties) or an oxidized carrier ampholyte buffer (see Righetti[16] for an extensive review on these chemicals). In fact, we have proved that in an IPG run, human α-globin chains (p*I* ≈ 9.2) were at least 50% oxidized in an overnight run, whereas if the IPG matrix were reduced in ascorbic acid prior to the run, full protection of globin chains would ensue[3]. The same was true in a CA–IEF gel: depending on the initial level of persulphate used, as much as 80% oxidation of α-chains could be elicited in only 4 h[5].

As we are dealing here with IPG gels, can in reality our presumptive $R_3N^+–O^-$ species oxidize thiols? In addition to the data presented here, there is ample evidence in the literature for such a mechanism. Thus, it is known that nitroso and nitro compounds can indeed oxidize thiols. In basic media, thiols are oxidized to disulphides by nitrobenzene or nitrosobenzene[17], which are in turn reduced mainly to azoxy- and azobenzene. Among the oxidizing species reported, 4-nitropyridine N-oxide (a weakly basic compound which could mimic grafted Immobilines) seems to be among the most reactive species[18].

Another aspect we should consider is the kinetics of the phenomenon: when incubated in the presence of oxidized IPG particles under aerobic conditions, full oxidation of Cys to cystine is seen to occur in 4 h. There are examples in the literature of even faster reactions. Thus, 2-propene-1-thiol is 90% oxidized by diethyl azodicarboxylate at room temperature in only 30 min[19]; 2-mercaptobenzothiazole is converted into the disulphide by the same reagent with 95% yield in 30 min at the refluxing temperature of benzene. We should, however, distinguish between oxidation kinetics under anaerobic or aerobic conditions. In the former instance, the kinetics are slowed by a factor of *ca.* 3 (full oxidation in 12 h anaerobically *vs.* 4 aerobically). Hence the presence of both oxidized IPG particles and atmospheric oxygen has a synergistic effect on the reaction. We also emphasize that in proteins Cys oxidation does not necessarily occur at the rate we have demonstrated here for free Cys. In fact (see Fig. 10 in ref. 3), under anaerobic conditions and with a reduced IPG

matrix, oxidation of Cys in α-globin chains is fully inhibited in an overnight run. Probably the protein moiety shields the Cys group and thus considerably slows the oxidation process.

Can a higher oxidation level be obtained in the presence of oxidized IPGs or CAs? At present we have only been able to see the conversion SH to -S-S-, whereas Jacobs[20] has reported oxidation to cysteic acid. Although in our HPLC system we cannot see any cysteic acid (which is well separated by our system; see Fig. 2), we cannot exclude the presence of other oxidation intermediates, as the TLC plate (see Fig. 1) gave a clear hint of two oxidized spots. Jacobs[20] reported oxidation of cysteine to cysteic acid in alkaline proteins, such as ribonuclease, after CA–IEF. As his data were based on total hydrolysis of ribonuclease isoforms, followed by chromatography with an amino acid autoanalyser, traces of CAs present in the protein during hydrolysis could have catalysed full oxidation to the cysteic acid form. It is known, in fact, that weak thiols can be partially transformed into their conjugated base by amines. As the anion oxidation proceeds much faster than on the undissociated thiol, this could account for Jacobs' data.

We conclude with the following guidelines for performing successful IPG fractionations of proteins:

(a) reduce the IPG gel by a washing step in 100 m*M* ascorbate (pH 4.5);

(b) run the IPG gel anaerobically (either submerged under paraffin oil or sandwiched between two plates);

(c) try to minimize the focusing time (4–5 h, instead of an overnight run).

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