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Note

The reduction of ferric myoglobin by Ampholine on acrylamide gel electrofocusing*

The ampholyte carrier system developed by VESTERBERG AND SVENSSON[1] and marketed under the trade name Ampholine by LKB-Produkter AB has become widely used for the fractionation of proteins by the technique of electrofocusing. The fractionation procedure initially utilized a sucrose density gradient for stabilization of the pH gradient. More recently, the use of polyacrylamide gel, as the anti-convection medium, was found[2] to have several advantages over density gradients.

VESTERBERG AND SVENSSON [1] and VESTERBERG[3] employed the density gradient procedure to fractionate and study ferrous and ferric multiple horse myoglobins. Using polyacrylamide gel electrofocusing in this laboratory[4] to characterize multiple bovine myoglobins it was observed that ferric myoglobins (Met-Mbs) underwent gradual reduction to their ferrous forms during the focusing procedure. In view of the natural tendency of myoglobins to autoxidize[5] and also, because of the known[6] oxidizing effects of persulfate in gels, the reduction reaction was unexpected and seemed worthy of exploration.

Materials and methods

Three batches of Ampholine were tested; No. 20, pH 6–8; No. 43, pH 6–8, and No. 48, pH 3–10. Ammonium persulfate, riboflavin, and 4,4,4′,4′-tetramethylethylenediamine (TMED) were purchased as reagent grade quality. Acrylamide and N,N′-methylenebisacrylamide were obtained commercially and purified before use by the procedure of LOENING[7].

The electrofocusing experiments were performed essentially as described by WRIGLEY[2]. Gel cylinders, 5 mm in diameter, consisted of 5% acrylamide, 2% Ampholine, 0.05% TMED, and either 0.0075% riboflavin or 0.035% ammonium persulfate. Samples of myoglobin (Mb), prepared as previously described[4], were oxidized with excess $K_3Fe(CN)_6$ and focused at 300 V (8 gel cylinders) at 4°.

After a focusing period the gels were removed from the focusing apparatus and, still in their quartz tubes, scanned spectrophotometrically[4]. The same gels were then stored at room temperature for various time periods, refocused, and scanned again. The change in the relative intensities of the ferric and ferrous Mb zones was recorded against time in the polyacrylamide gel. The ferric and ferrous zones were identified by their absorbance curves in the visible spectrum and, also, by their isoelectric points (*p*I).

The reduction reaction was also studied in the absence of polyacrylamide. The changes in absorption spectra between 470 and 630 nm of solutions containing Mb, riboflavin (1.9×10^{-4} *M*) or persulfate (5×10^{-3} *M*), Ampholine (2%), and TMED (0.12%) were recorded against time.

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Results and discussion

The gradual reduction of Met-Mb with time in the Ampholine–persulfate–polyacrylamide gel is demonstrated in Fig. 1. Zone A with a *p*I of 7.05 (25°) has the absorption spectrum of Met-Mb, while zone B with a *p*I of 6.84 exhibits the spectrum of ferrous Mb. The ratio zone B: zone A is seen to increase with time. The reducing capacity of the gels is eventually expended and the reduced Mb slowly reverts to its ferric form. The extent of reduction and the time of onset of re-oxidation is affected by pre-electrolysis of the gels; that is, focusing the gels before sample loading diminishes its reducing capacity. Pre-electrolyzing for up to 3 h, however, was insufficient for eliminating that capacity.

Spectral analyses of zone B indicate it to be a mixture of the oxygenated and deoxygenated forms of the ferrous protein. The proportion of oxy-Mb in the zone

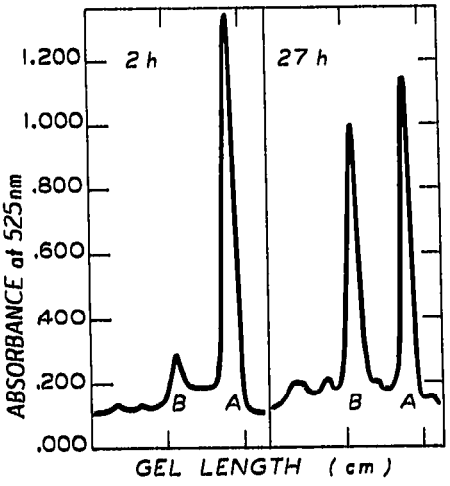

Fig. 1. Spectrophotometric scans of an electrofocused myoglobin sample after various periods in a polyacrylamide gel.

increases with time, supposedly as oxygen diffuses into the gel. Degasing the acrylamide solution prior to gelation results in a decrease in the initial proportion of oxy-Mb.

The reduction reaction occurs much faster in gels polymerized by the riboflavin system rather than by persulfate. Regardless of the polymerizing system employed, however, an excess of $K_3Fe(CN)_6$ added to the cathode chamber results in the disappearance of the ferrous zone.

The reducing effect was observed, not only in gels, but also in acrylamide-free solution. Fig. 2 depicts the spectral changes of Met-Mb incubated with Ampholine and persulfate. The spectrum changes from the absorption curve of Met-Mb (¼ h) to a curve exhibiting mainly oxy-Mb (1¼ h). The 8-h curve demonstrates the gradual re-oxidation of the reduced Mb. Samples incubated in Ampholine without persulfate or in the presence of persulfate without Ampholine (substituting 0.05 *M* Tris, pH 7.0, buffer) exhibited no spectral changes. TMED had no apparent effect on the changes.

Qualitatively similar results were obtained on replacing persulfate with ribo-

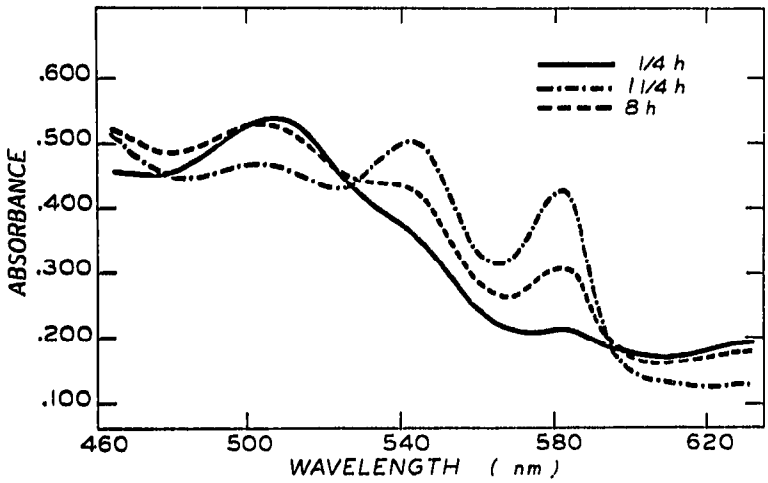

Fig. 2. Absorption spectra at various time intervals of a myoglobin sample incubated with Ampholine and persulfate.

flavin in the incubation solutions. Thus, in summary, ferric Mb is reduced by the combination of Ampholine and either persulfate or riboflavin.

Persulfate has been implicated as a source of spurious protein band formation in polyacrylamide gel electrophoresis[6] and electrofocusing[8]. The heterogeneity observed with Ampholine, however, is not caused by protein denaturation, but solely by change of valency of the heme iron.

The change of heme iron valency is readily identified by spectrophotometry and may be avoided simply by focusing in the presence of excess $K_3Fe(CN)_6$ and/or KCN (ref. 4). Thus, once recognized, there is no problem with Mb. However, the fact that a reducing environment is created when Ampholine is used in polyacrylamide gels could have significance to the electrofocusing of other protein systems and as such should be noted.

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