THE ROLE OF THE REDUCING AGENT IN THE ACTIVATION OF ACONITASE*

by

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INTRODUCTION

Various investigators have shown that aconitase is activated by ferrous ions and a reducing agent[1-4]. The enzyme is not activated by other metallic ions, although ascorbate, glutathione or cysteine can be used as reducing agents[2]. We have observed that hydroxylamine also can be used as a reducing agent. DICKMAN AND CLOUTIER[2] have suggested that the reducing agent functions by keeping iron and the protein in the reduced state. MORRISON[4] assumes that a protein-metal-reducing agent complex is the active form of aconitase.

A very sensitive method for the determination of aconitase activity is that of measuring the rate of formation of aconitate at 240 mμ using either citrate or *iso*-citrate as substrate[5]. While using this method for the determination of aconitase activity, we observed an increased absorbance at 240 mμ when ferrous ions were added to citrate in the absence of enzyme. MORRISON AND PETERS[6] also observed this non-enzymic reaction. Upon further examination of this phenomenon we found that this non-enzymic absorbance, resulting when ferrous ions were added to citrate, was a measure of chelate formation. Also, we observed that accompanying this increased absorbance ferrous ions were oxidized to ferric ions. These observations suggested a means of studying the iron citrate system and a possible role of the reducing agent in the activation of aconitase.

MATERIALS AND METHODS

All reagents were c.p. and were made up fresh before each determination.

Absorption spectra were obtained using the Beckman Model DU spectrophotometer and pH determinations were made with the Beckman Model G pH meter. The citrate ($Na_3C_6H_5O_7 \cdot 1/2 H_2O$) solution was adjusted to pH 4.6 before adding the metal. The stock iron ($FeSO_4 \cdot 7H_2O$) solutions and all dilutions were made with glass-distilled water, pH 4.6. It was found that the pH varied less than a 0.1 of a pH unit over a twenty-four hour period. The iron, citrate and iron citrate mixtures were stored in the dark at room temperature.

Iron determinations were carried out according to the method of SANDELL[7] using *o*-phenanthroline and hydroxylamine. The pH was 4.0 as suggested by HARVEY *et al.*[8] and the *o*-phenanthroline, hydroxylamine, buffer (1 *M* acetate) and sample were incubated at 25° C for thirty minutes before determining the absorbance at 500 mμ.

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RESULTS AND DISCUSSION

Initial observations on this non-enzymic phenomenon occurring when ferrous ions were added to citrate indicated that the increase in absorbance at 240 mμ was proportional to the amount of added ferrous ions. Ferrous ions at levels of 0.10, 0.07, 0.05, 0.03, and 0.01 μ*M* when added to 0.8 μ*M* of citrate produced a change in optical density in one minute of 0.209, 0.160, 0.108, 0.052, and 0.028, respectively. Also, we observed that the presence of reducing agents had an inhibitory effect on the increased absorbance at 240 mμ. Nickel, cobalt, magnesi m manganese, zinc and calcium did not show an increase in absorbance at 240 mμ when added to citrate. *Iso*-citrate, *cis*-aconitate, *trans*-aconitate, glycine, tris buffer and pyrophosphate can be substituted for citrate and show increased absorbance at 240 mμ when added to ferrous ions. By chemical and enzymic tests it was not possible to show that citrate and aconitate were being interconverted, nor was it possible to show that citrate was disappearing, yielding a product other than aconitate.

The above results were obtained at pH 7.4, the reported pH optimum of aconitase. At this pH ferrous ions in the iron citrate system were rapidly oxidized to ferric ions. In order to determine whether this oxidation was inherent to chelate formation or solely to the pH, a study of the iron citrate system was undertaken at pH 4.6.

It was found that at pH 4.6 the increase in absorbance is proportional to the oxidation of ferrous ions, within experimental error. These data are shown in Table IA and B. Table IA shows the proportionality between the absorbance at 240 mμ and the relative concentrations of ferrous and ferric ions for a 1:1 mixture of pH 4.6 at varying time intervals. The ratio 1:1 indicates the micromoles of ferrous ions:

TABLE I

PROPORTIONALITY BETWEEN THE INCREASE IN ABSORBANCE AT 240 mμ AND THE OXIDATION OF FERROUS IONS AT pH 4.6

In Table IA, 0.1 μ*M* Fe^{++} were added to 0.1 μ*M* citrate and in Table IB 0.054 μ*M* Fe^{++} were added to 0.162 μ*M* citrate.

Time (hours)	*Optical density 240 mμ*	*μM Fe++*	*μM Fe+++*
		A	
0	0	0.100	0.00
1	0.195	0.075	0.033
2	0.251	0.068	0.040
3	0.282	0.065	0.043
7	0.330	0.054	0.054
24	0.410	0.046	0.062
48	0.321	0.046	0.062
24 (Iron blank)	0.006	0.100	0.00
		B	
0	0	0.054	0.00
1	0.136	0.036	0.018
2	0.193	0.025	0.032
3	0.215	0.021	0.034
7	0.279	0.018	0.036
24	0.308	0.012	0.042
48	0.321	0.012	0.042
24 (Iron blank)	0.005	0.054	0.00

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micromoles of citrate. In Table IB the results for a 1:3 mixture indicate that the proportionality between the increase in absorbance and the oxidation of ferrous ions during the initial reaction is not as striking as that reported for the 1:1 mixture. It should be noted in Table IA that the absorbance decreases from 24 to 48 hours, but the concentrations of ferrous and ferric ions remain constant. In Table IB the optical density increases slightly from 24 to 48 hours, but the relative iron concentrations remain constant. This slight increase in absorbance from 24 to 48 hours for the 1:3 mixture was observed only in a few determinations; usually a decrease was observed. Also, it should be noted in Table IA and B that the iron blanks showed no oxidation at this pH. Therefore, it is unlikely that the oxidation of ferrous ions in the presence of citrate is due to dissolved oxygen.

At pH 4.6 the method of continuous variation indicated that ferrous ions and citrate combine in a ratio of 1:1.

Total iron and ferrous iron concentrations were determined using *o*-phenanthroline. With this method the ferric ion concentration is obtained by difference. The possibility arises that citrate interferes with the iron assay and that the measured "ferric" ion concentration is actually the concentration of ferrous ions chelated with citrate. However, with the data available at the present time it is doubtful that this is the case in view of the large excess of *o*-phenanthroline that is added for the assay of iron. Further evidence that the concentration of citrate, used in these experiments, does not interfere with the *o*-phenanthroline tests is to be found in the work of HARVEY *et al.*[8] and FORTUNE AND MELLON[9]. These investigators have found independently that a ten-fold excess of citrate over iron will not interfere with the *o*-phenanthroline test. HARVEY *et al.*[8] report interference when citrate is present at an excess of 125-fold that of iron. This concentration of citrate was greater than the concentration of added *o*-phenanthroline. For the experiments discussed in this paper, the concentration of citrate never exceeded the iron concentration by ten fold. Hence, it is doubtful that citrate is interfering with the *o*-phenanthroline test.

Conclusive evidence that ferrous ions are oxidized to ferric ions at pH 4.6 in the presence of citrate could be obtained by polarographic determinations. Qualitative evidence supporting the postulate that ferrous ions are oxidized to ferric ions at pH 4.6 in the presence of citrate was suggested by the finding of positive tests for ferric ions with ferrocyanide and thiocyanate in the presence of dilute hydrochloric acid. In addition to this qualitative evidence we have observed that in the presence of a reducing agent, hydroxylamine, the iron-citrate mixture shows no absorbance at pH 4.6. Also, we have observed that bubbling nitrogen through the iron citrate mixture immediately after adding the metal to the chelating agent produces an effect similar to that of adding a reducing agent.

When hydroxylamine at a final concentration of 0.2% is added to an iron citrate mixture that has stood for 24 hours, the absorbance decreases to zero and all the iron is present as ferrous ions. These results are presented in Table II and indicate that hydroxylamine will reduce the iron to the divalent state. Whether or not the reducing agent displaces citrate from the iron cannot be stated at the present time.

When 0.1 μM ferric ions are added to 0.1 μM citrate at pH 4.6, the spectrum ten minutes after mixing the metal with the chelating agent is almost identical to the one obtained after twenty-four hours. The spectrum increases approximately 10% during this time interval in contrast to the marked changes occurring during twenty-

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TABLE II

EFFECT OF ADDING HYDROXYLAMINE TO A 1:1 IRON CITRATE SYSTEM (pH 4.6) 24 HOURS AFTER MIXING THE METAL AND CHELATING AGENT

Time (minutes) after adding hydroxylamine	*Optical density 240 mμ*	*μM Fe++*	*μM Fe+++*
0	0.395	0.048	0.052
1	0.283		
2	0.230		
3	0.198		
4	0.170		
5	0.150		
10	0.083		
30	0.024		
60	0.023	0.100	0.00

four hours when ferrous ions are added to citrate. The ultraviolet absorbance spectrum of ferric ions and a ferric citrate mixture are shown in Fig. 1. The spectra of a ferrous citrate mixture, ferrous ions and citrate are included in this figure. When these curves are corrected, it can be seen that the ferric citrate spectrum obtained by adding ferric ions to citrate is almost identical to the spectrum obtained when ferrous ions are added to citrate. The corrected curve obtained by adding ferric ions to citrate always showed slightly less absorption than the one obtained by adding ferrous ions to citrate possibly due to greater dissociation.

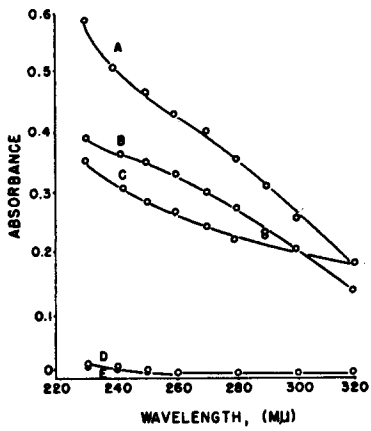

Fig. 1. Absorption spectra of iron citrate systems at pH 4.6 twenty-four hours after mixing the metal and chelating agent. Curve A, 0.1 μM Fe^{+++} + 0.1 μM citrate; Curve B, 0.1 μM Fe^{++} + 0.1 μM citrate; Curve C, 0.1 μM Fe^{+++}; Curve D, 0.1 μM citrate and curve E, 0.1 μM Fe^{++}. Iron analysis of the mixture represented in curve B showed that of the original 0.1 μM Fe^{++} added to citrate, 0.060 μM Fe^{+++} and 0.042 μM Fe^{++} were present after 24 hours.

From the results presented above it is postulated that when ferrous ions are added to citrate at pH 4.6, a ferrous citrate chelate is formed. The iron in this chelate is oxidized to ferric ions and this ferric citrate chelate absorbs at 240 mμ.

It seems likely that the primary function of the reducing agent in aconitase activation is to maintain iron in the divalent state. Ferrous ions in the aconitase reaction would be oxidized by virtue of the ferrous substrate chelate and also by the pH. The concentration of reducing agent used with iron in activation studies of aconitase is not sufficient to maintain all the iron as ferrous ions. Although no direct determination of iron concentration can be applied to this system due to the high concentration of chelating agents normally present, it is known that increased absorbance results at 240 mμ when the metal, citrate and reducing agent are mixed at the concentrations used for aconitase activation. MORRISON's[4] results on the increased activity of aconitase with increasing reducing agent concentration may be due primarily to more iron being maintained as ferrous ions. However, the results

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presented in this paper do not disprove MORRISON's[4] postulate of active aconitase being a protein-metal-reducing agent complex.

It is tempting to suggest that a primary function of reducing agents in the ferrous ion activation of other enzyme systems[10-12] may be quite similar to that postulated for aconitase activation.

SUMMARY

When ferrous ions are added to citrate, there is an increased absorbance at 240 mμ. Paralleling this increase in optical density, ferrous ions are oxidized to ferric ions at pH 4.6. The same results were obtained at pH 7.4. The presence of a reducing agent will inhibit this non-enzymic reaction at pH 4.6 and pH 7.4.

The application of these data to aconitase activation suggests that the primary function of the reducing agent is to maintain iron in the divalent state.

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