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Studies on Adrenal Steroid Hydroxylases. Reactivity of Iron Atoms in Adrenal Iron–Sulfur Protein (Adrenodoxin) with Iron-Chelating Agents*

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ABSTRACT: Kinetic studies on the reaction of adrenodoxin with iron chelators have been carried out in order to elucidate the reaction mechanism. The reaction rate of adrenodoxin with excess *o*-phenanthroline apparently follows zero-order kinetics under aerobic conditions, whereas it is a pseudo-first-order reaction under anaerobic conditions. The activation energies are 3.3, and 12.6 kcal per mole under aerobic and anaerobic conditions, respectively. Model experiments with inorganic compounds showed that the reaction of dithionite with Fe^{3+}–*o*-phenanthroline occurs about 400 times faster than with free Fe^{3+} ions, and that the reaction of *o*-phenanthroline with Fe^{2+} is very fast. In the presence of 4 M urea and 1 M KCl, the iron chromophore of adrenodoxin is quite stable, and the rate is faster than that of free Fe^{3+} ions at 26°. From these results, the reaction sequence is proposed to consist of at least binding of *o*-phenanthroline to the protein and subsequent reduction of Fe^{3+}–*o*-phenanthroline to its Fe^{2+} complex by the intramolecular reductants. The former reaction is affected by chaotropic agents, and the latter is sensitive to molecular oxygen. In the presence of chaotropic agents, Tiron reacts with all of the iron atoms in adrenodoxin to produce its Fe^{3+} complex. The Fe^{3+}–Tiron formed is not reduced by the intramolecular reductants. Accordingly, the reaction is not affected by molecular oxygen. These results are consistent with the implication that all of the iron atoms in adrenodoxin are ferric. In addition to this, the iron atoms in adrenodoxin are less accessible to the chelating agents than those in bacterial ferredoxin.

In general, ferredoxin serves as a redox intermediate of electron-transfer reactions in a variety of physiological systems. A ferredoxin-like protein, adrenodoxin, is a redox intermediate in the NADPH-dependent steroid hydroxylases of adrenal mitochondria. This protein has 2 g-atoms of iron and 2 moles of labile sulfur per mole of protein. The detailed properties have been reviewed previously (Kimura, 1968a).

The reactivity of clostridial ferredoxin with iron-chelating agents has been studied to some extent by Malkin and Rabinowitz (1967). Their work showed that the iron atoms are not readily accessible to chelating agents; however, in the presence of molecular oxygen or chaotropic agents these reagents chelate the iron atoms in ferredoxin.

There are some similarities and dissimilarities among ferredoxins in terms of their iron contents, amino acid sequences, and valence states of iron. Thus, it is of interest to compare the chelator accessibility of adrenodoxin with that of bacterial ferredoxin. Furthermore, three recent physical approaches to the iron valence state on ferredoxins strongly suggest that both iron atoms in adrenodoxin and spinach ferredoxin are ferric (Eaton *et al.*, 1971; Johnson *et al.*, 1971; Poe *et al.*, 1971), whereas 6–8 iron atoms in bacterial ferredoxin are mixtures of Fe^{3+} and Fe^{2+} ions (Blomstrom *et al.*, 1964).

Because in the previous studies by Malkin and Rabinowitz (1967) the authors had difficulty interpreting their results due to lack of information on the valence states of iron atoms in ferredoxin, and also due to the multiplicity of iron content and valence states, it is timely to reinvestigate the reactivity of adrenodoxin with iron chelators. In this paper, some newer interpretations on the reactivities will be presented.

Materials and Methods

Bovine adrenodoxin was prepared as described previously (Kimura, 1968a). The ratio of A_{414} to A_{276} of the preparations used in this investigation was 0.76–0.83, and the iron content was 1.9 g-atoms of iron/mole of protein, assuming a molecular weight of 13,000. The purity of the sample was regarded as more than 90%. An NADPH diaphorase (adrenodoxin reductase) was prepared by the method reported elsewhere (Kimura and Suzuki, 1967).

Urea, guanidine·HCl (Ultra Pure grade), *o*-phenanthro-

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TABLE I: Rate Constants of the Reaction of Adrenodoxin with Iron Chelators.[a]

Iron–Sulfur Protein	Chelator	Gas Phase	Addn	Temp (°C)	Zero-Order Rate Constant ($M\ min^{-1} \times 10^{6}$)	First-Order Rate Constant ($M\ min^{-1} \times 10^{2}$)
AD	OP	Air	None	25	0.34	
AD	OP	Air	None	39	0.40	
AD	OP	Air	None	49	0.58	
AD	OP	Air	G	23	(1.04)	3.84[b]
AD	OP	Air	U	23		0.61[b]
AD	OP	N_2	None	25	(0.06)	0.22[b]
AD	OP	N_2	None	39		0.47[b]
AD	OP	N_2	None	49		0.99[b]
AD	OP	N_2	U	38		0.22[b]
AD	T	Air	None	29	0	0
AD	T	Air	G	29		0.30[b]
AD	T	Air	U	29		0.22[b]
AD	T	N_2	None	29	0	0
AD	T	N_2	G	29		0.20[b]
AD	T	N_2	U	29		0.14[b]
FD	OP	N_2	None	*c*	0.27	
FD	OP	N_2	G			24
FD	OP	N_2	U			10
FD	T	O_2	G			12

[a] The reaction mixture for the *o*-phenanthroline reactions contained: 3.69×10^{-5} M adrenodoxin (in terms of iron), 3.12×10^{-4} M *o*-phenanthroline, 0.01 M phosphate buffer (pH 7.4), and 6 M urea or 3 M guanidine·HCl, if added. The mixture for the Tiron reactions contained: 7.38×10^{-5} M adrenodoxin (in terms of iron), 5.89×10^{-4} M Tiron, 0.5 M Tris buffer (pH 7.4), and 6 M urea or 3 M guanidine·HCl, if added. The values of ferredoxin were taken from the data of Malkin and Rabinowitz (1967). The values in parentheses are calculated from the first-order rate constants for convenient comparison with other experiments. The abbreviations used are: AD, adrenodoxin; FD, ferredoxin; OP, *o*-phenanthroline; T, Tiron; G, guanidine·HCl; U, urea. [b] The order of reaction is complex. [c] The exact temperature was not described in the reference (Malkin and Rabinowitz, 1967).

line, Tiron,[1] and NADPH were obtained from Fisher, Mann, Smith, Fisher, and Sigma, respectively. *m*-Phenanthroline was a gift of Dr. O. Hayaishi. Spectrophotometric measurements were carried out by the use of Hitachi automatic recording spectrophotometers (EPS-3 and 124). Stopped-flow measurements were performed with a Durrum-Gibson spectrophotometer. O_2 uptake was measured by a rotating oxygen electrode. Determinations of protein, iron, and labile sulfur were as described previously (Kimura and Suzuki, 1967). For calculations, the following molar extinction coefficients were used: ϵ 11.3×10^3 M^{-1} cm^{-1} at 510 nm (Fe^{2+}–*o*-phenanthroline), ϵ 5.3×10^3 M^{-1} cm^{-1} at 490 nm (Fe^{3+}–Tiron), ϵ 4.9×10^3 M^{-1} cm^{-1} at 414 nm (iron in adrenodoxin), ϵ 2.7×10^3 M^{-1} cm^{-1} at 510 nm (iron in adrenodoxin), and ϵ 3.2×10^3 M^{-1} cm^{-1} at 490 nm (iron in adrenodoxin). The rate constants are expressed in terms of iron in adrenodoxin.

Results

Formation of Fe^{2+}–o-Phenanthroline from Oxidized Adrenodoxin. The optical absorption spectrum of oxidized adrenodoxin changed upon reaction of the iron with *o*-phenanthroline under aerobic and anaerobic conditions, undoubtedly indicating that octahedral iron–*o*-phenanthroline complex was produced from iron atoms in this protein. *m*-Phenanthroline, a structural analog but not a metal chelator, had no effect on the optical spectrum of oxidized adrenodoxin.

o-Phenanthroline reacts slowly with the iron in oxidized adrenodoxin in the absence of oxygen at several temperatures. Upon aeration, the rate of formation of Fe^{2+}–*o*-phenanthroline is considerably increased, being similar to the case of bacterial ferredoxin. The rate nicely follows pseudo-first-order kinetics under anaerobic conditions, whereas it is apparently zero order under aerobic conditions. The initial rates of the reactions do however not follow the above kinetics.

The effects of temperatures from 17 to 49° on the rate of the formation of Fe^{2+}–*o*-phenanthroline from oxidized adrenodoxin were examined during later times of reaction. The Arrhenius plots yield the activation energies of 12.6 and 3.3 kcal per mole under anaerobic and aerobic conditions, respectively. Molecular oxygen decreases the energy by about 10 kcal/mole.

Effects of Urea and Guanidine·HCl on the Formation of Fe^{2+}–o-Phenanthroline and Fe^{3+}–Tiron from Oxidized Adrenodoxin. The aerobic reaction of *o*-phenanthroline with oxidized adrenodoxin was markedly stimulated by 3 M guanidine·HCl or 6 M urea. All of the iron atoms in adrenodoxin reacted with *o*-phenanthroline to produce the Fe^{2+} complex in the presence of these denaturants. In contrast to this, the anaerobic reaction was inhibited by urea. The reaction of Tiron with oxidized adrenodoxin was also stimulated by the addition of chaotropic agents under either aerobic or anaerobic conditions. In the presence of guanidine·HCl, all of the iron atoms reacted with Tiron to produce its Fe^{3+} complex under both aerobic and

[1] Abbreviation used is: Tiron, 4,5-dihydroxy-*m*-benzenedisulfonic acid disodium salt.

TABLE II: The Fe^{2+}–*o*-Phenanthroline and Fe^{3+}–Tiron Formation in the Presence of 4 M Urea and 1 M KCl.[a]

Solvent	Gas Phase	Temp (°C)	Experiment I[b]		Experiment II[b]		Experiment III[b]
			Fe^{2+}–*o*-Phenanthroline Formation	O_2 Uptake	Fe^{3+}–Tiron	O_2 Uptake	Denaturation of Adrenodoxin[c]
Urea	O_2	27	0.79	0[d]	0.06		0.20
Urea	O_2	36	1.16	0[d]	0.10		0.54
Urea + KCl	O_2	27	10.8	13.6	0.06	0[d]	0.00
Urea + KCl	O_2	36	22.8	29.0	0.12	0[d]	0.11
Urea	N_2	24	0.26				
Urea + KCl	N_2	37	5.3[e]		0.14		

[a] The reaction mixtures contained 1.14×10^{-5} M adrenodoxin (in terms of iron) in 4 M urea containing 0.01 M phosphate buffer (pH 7.4), or in 4 M urea containing 1 M KCl and 0.01 M phosphate buffer (pH 7.4). If added, *o*-phenanthroline or Titron contained at 3.34×10^{-4} M. The aerobic reactions with urea without KCl displayed a complex order of reaction. The rates were calculated from the linear part of the curves. [b] In M min^{-1}. [c] Denaturation of adrenodoxin was measured by chanes in optical absorbance at 414 nm in the absence of iron chelators. [d] O_2 uptake could not be detected even a prolonged time of reaction, presumably being due to the slow reaction. [e] The rate was calculated from the first-order rate constant.

anaerobic conditions. As noted, the rate of the Tiron reaction was not affected by O_2. (The values in Table I are regarded as within experimental errors.)

In Table I, the rate constants of the reaction of *o*-phenanthroline or Tiron with oxidized adrenodoxin are summarized and compared with the values obtained with clostridial ferredoxin (Malkin and Rabinowitz, 1967).

Reactivity of o-Phenanthroline and Tiron with Oxidized Adrenodoxin in the Presence of 2 M Urea and 1 M KCl. Petering and Palmer (1970) observed that the denaturation of the iron chromophore of spinach ferredoxin by urea was largely prevented by a high ionic strength medium. A similar phenomenon was also observed in the case of adrenodoxin. We have carried out the reaction of *o*-phenanthroline and Tiron with adrenodoxin in 4 M urea and 1 M KCl solution under aerobic conditions, where the iron chromophore is stable enough, but the polypeptide chain is supposed to be unfolded. The rate of the Fe^{2+}–*o*-phenanthroline formation in the presence of 1 M KCl was about 10–20 times faster than in the absence of KCl. In complete contrast to this, the Tiron reaction was not affected by the addition of KCl. In the presence of 4 M urea and 1 M KCl, the *o*-phenanthroline reaction consumed molecular oxygen at a rate comparable to that of the Fe^{2+}–*o*-phenanthroline formation. In 4 M urea under anaerobic conditions, the rate of *o*-phenanthroline reaction was found to be about four times slower than that under aerobic conditions regardless of the presence of 1 M KCl. These results are shown in Table II.

Comparison of Adrenodoxin with Inorganic Compounds in the o-Phenanthroline Reaction. The initial rates of formation of Fe^{2+}–*o*-phenthroline from oxidized adrenodoxin were measured by the use of a stopped-flow spectrophotometer. The order of the initial reaction was found to be complex in contrast to later times of reaction: zero-, first-, or second-order kinetic curves could not be fitted to the experimental curve. In contrast, the rate of adrenodoxin–*o*-phenanthroline formation in 4 M urea and 1 M KCl solution was nicely fitted to a pseudo-first-order kinetics. Table III lists the initial rates of the *o*-phenanthroline reactions with adrenodoxin samples and inorganic compounds. These results show that the reaction of Fe^{2+} ions with *o*-phenanthroline is very fast, and the reduction of Fe^{3+}–*o*-phenanthroline is also fast, whereas that of Fe^{3+} ions is slow. From the model experiments, it may be stated that the rate-limiting step in the Fe^{2+}–*o*-phenanthroline formation is the reaction of Fe^{3+} ions with *o*-phenanthroline. When the reaction of adrenodoxin samples is compared with that of Fe^{3+} ions, their rates have a comparable order of magnitude which is quite different from the other model systems. Furthermore, the reaction of adrenodoxin in 4 M urea and 1 M KCl was found to be faster than that of free Fe^{3+} ions but slower than that of Fe^{2+} ions, indicating that under this condition *o*-phenanthroline is freely accessible to the iron atoms in adrenodoxin.

Reactivity of o-Phenanthroline with Reduced Adrenodoxin. The reactivity of *o*-phenanthroline with dithionite-reduced adrenodoxin was examined. As illustrated in Figure 1, the reduced protein which exhibits a new peak at 540 nm, reacts with *o*-phenanthroline at a rate comparable to that of the oxidized protein under anaerobic conditions. It therefore appears that the reduced and oxidized proteins have similar accessibilities of iron to *o*-phenanthroline.

Enzymatic Reduction of Fe^{3+}–o-Phenanthroline by NADPH and Adrenodoxin Reductase. As shown in Figure 2, adrenodoxin reductase, an NADPH diaphorase, can reduce the externally added Fe^{3+}–*o*-phenanthroline to the Fe^{2+}–*o*-phenanthroline, depending on the enzyme amounts added. There was no stimulation by the addition of adrenodoxin.

The reduction of the Fe^{3+} complex by the reductase can be explained by the oxidation–reduction potential of the complex and also by the fact that the reductase has a wide specificity toward electron acceptors such as dichlorophenol indophenol, cytochrome *c*, ferricyanide, vitamin K, and dinitrophenol (Kimura, 1966, 1968b). Since the iron atoms in reduced adrenodoxin is not readily accessible to *o*-phenanthroline, adrenodoxin cannot stimulate the rate of the Fe^{2+}–*o*-phenanthroline formation.

Therefore, the formation of Fe^{2+}–*o*-phenanthroline by enzymatic reduction, in general, does not necessarily reflect the original valence state of iron atoms of proteins, because any contaminating Fe^{3+} ions in the protein samples, if there are, can thus be reduced in the presence of *o*-phenanthroline.

TABLE III: Initial Rates of the Reaction of *o*-Phenanthroline with Oxidized Adrenodoxin.[a]

Expt	Reactants: Syringe A	Reactants: Syringe B	Zero-Order Rate at 25° (M min^{-1})	First-Order Rate at 25° (min^{-1})
I	Fe^{2+} (8 μM)	OP (0.2 mM)	(4.8×10^{-3})	1.2×10^{3}
II	Fe^{3+} (8 μM)	OP (0.2 mM)–dithionite (4 mM)	3.1×10^{-6}	
III	Fe^{3+} (8 μM)-OP (0.2 mM)	Dithionite (4 mM)	1.3×10^{-3}[b]	
IV	Adrenodoxin (14×10^{-5} M)	OP (2 mM)	1.2×10^{-6}[b]	
V	Denatured adrenodoxin (14×10^{-5} M)	OP (2 mM)	$6\text{–}25 \times 10^{-6}$	
VI	Adrenodoxin in 4 M urea and 1 M KCl (14×10^{-5} M)	OP (2 mM)	(1.5×10^{-5})[c]	3.7

[a] The rate of the formation of Fe^{2+}–*o*-phenanthroline was measured at 510 nm by the use of a stopped-flow spectrophotometer. In expt V, heat-denatured adrenodoxin was prepared as follows: the heat treatment was carried out for 21.5 hr at 45° under anaerobic conditions. The characteristic color of adrenodoxin disappeared by this treatment. After passing through a Sephadex G-25 column, 79% of the total iron atoms were found to be bound to the protein and 80% or more of the total labile sulfur was removed. All of the bound iron atoms then reacted with *o*-phenanthroline to produce its Fe^{2+} complex. OP represents *o*-phenanthroline. The values in parentheses are calculated from the first-order rate constant for comparison. [b] The order of reaction is complex. [c] This rate is a calculated value, when the concentration of Fe^{3+} ions is 4×10^{-6} M at the reaction mixture. This faster rate compared with that of free Fe^{3+} ions (expt II) is, perhaps, due to the exchange reaction of Fe^{3+} ions from the iron-sulfur group in adrenodoxin to *o*-phenanthroline.

Discussion

In spite of considerable effort on the part of many investigators, the valence states of the iron atoms in oxidized and reduced ferredoxins have not been well defined. However, recent physical approaches to the valence states of adrenodoxin and spinach ferredoxin strongly suggest that both iron atoms in the oxidized proteins are ferric and upon reduction one of the two iron atoms is reduced to ferrous ion and the other remains as ferric (Eaton *et al.*, 1971; Johnson *et al.*, 1971; Poe *et al.*, 1971).

Although the data presented in this paper do not completely establish the valence state of iron, particularly the abnormal valence state, all experimental evidence obtained is quite consistent with the implication that the valence state of all of the iron atoms in oxidized adrenodoxin are formally ferric. The following lines of evidence strongly support this idea. (1) In the presence of chaotropic agents, Tiron reacts

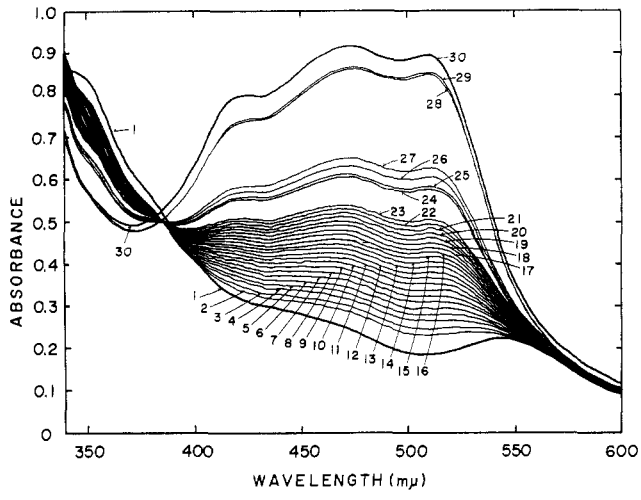

FIGURE 1: Reaction of *o*-phenanthroline with reduced adrenodoxin. The reaction mixture contained: 1.45×10^{-4} M reduced adrenodoxin (in terms of iron), 1.45×10^{-3} M *o*-phenanthroline, and 0.01 M phosphate buffer (pH 7.4). The reduced protein was prepared by titrating with standard dithionite solution under anaerobic conditions, and then the reaction was started by adding *o*-phenanthroline. All manipulations were carried out under N_2 atmosphere. Curve 1, reduced adrenodoxin; curve 2, 3 min after initiation of the reaction; curves 3–26, at 10-min intervals after curve 2; curves 24–27, 290, 300, 330, and 360 min, respectively; curve 28, 16 hr; curve 30, after 16 hr, air was introduced. After 16-hr anaerobic reaction, the Fe^{2+}–*o*-phenanthroline formed was calculated to be 226 nmoles which is 52% of the total iron atoms in adrenodoxin.

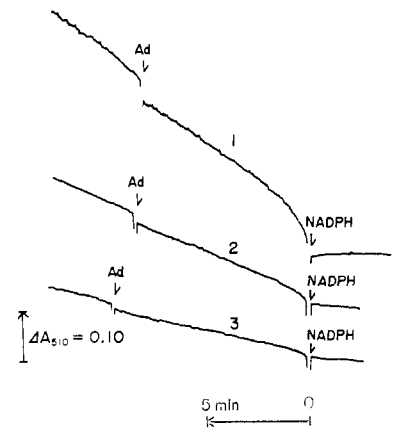

FIGURE 2: Enzymatic reduction of ferric *o*-phenanthroline by adrenodoxin reductase. The reaction mixture contained: 0.100 ml (curve 1), 0.075 ml (curve 2), and 0.050 ml (curve 3) of adrenodoxin reductase, and 1 μmole of Fe^{3+}–*o*-phenanthroline which was prepared from $Fe(NO_3)_3$ and *o*-phenanthroline. At the first arrows, 0.5 μmole of NADPH was added, and the second arrows, 19.7 natoms of iron in adrenodoxin was added. The reaction was carried out at 25° under aerobic conditions. The increase in optical absorbance was recorded at 510 nm, and the total reaction mixture had a volume of 3.00 ml.

with all of the iron atoms in this protein to produce the Fe^{3+} complex, (2) the rate of the formation of Fe^{2+}–*o*-phenanthroline from adrenodoxin in the absence of chaotropic agents has a rate most similar to that of Fe^{3+} ions among the other model systems, (3) the rate of reaction in the presence of guanidine·HCl becomes nearly comparable to that of free Fe^{3+} ions, (4) the rate in the presence of 4 M urea and 1 M KCl is faster than that of free Fe^{3+} ions, but slower than free Fe^{2+} ions, and (5) one of the two iron atoms in the reduced protein reacts with *o*-phenanthroline faster than the other, being consistent with one-electron reduction of this protein (Kimura *et al.*, 1970).

Thus, it would be reasonable to propose mechanisms 1 and 2.

$$\text{adrenodoxin–}(2Fe^{3+}) \xrightarrow[k\,=\,10^{-5}\ \text{M min}^{-1}]{\text{o-phenanthroline}}$$

$$\text{adrenodoxin–}(2Fe^{3+})\text{–o-phenanthroline} \xrightarrow[k\,=\,10^{-3}\ \text{M min}^{-1}]{\text{intramolecular reductants}} \text{adrenodoxin–}(2Fe^{2+}\text{–o-phenanthroline}) \quad (1)$$

$$\text{adrenodoxin–}(2Fe^{3+}) \xrightarrow[k\,=\,10^{-7}\ \text{M min}^{-1}]{\text{Tiron}} \text{adrenodoxin–}(2Fe^{3+}\text{–Tiron}) \quad (2)$$

The first step is rate limiting in overall reaction. Chaotropic agents perhaps accelerate this first step. In order to explain the slow reaction rate of the native oxidized protein compared with the model system, one must assume at least the two partial reactions, binding of *o*-phenanthroline to the protein, and transfer of the protein-bound *o*-phenanthroline to the iron-binding site. The migration of *o*-phenanthroline molecules in polypeptide matrix is a slowest one, and this kinetic barrier can largely be removed by chaotropic agents.

When *o*-phenanthroline is a reactant, the second step in reduction of the Fe^{3+}–*o*-phenanthroline is sensitive to molecular oxygen (*vide infra*). When Tiron is a reactant, Fe^{3+}–Tiron formed is not reduced by the intramolecular reductants, presumably being due to the difference in oxidation–reduction potentials. Accordingly, the reactivity of Tiron is not affected by the presence of oxygen.

Since original observations of the oxygen effects on Fe^{2+}–*o*-phenanthroline formation from bacterial ferredoxin by Malkin and Rabinowitz (1967), the detailed mechanism has not been well understood. There appears to be at least the following two explanations. Upon reaction with molecular oxygen, a radical is produced which is more effective in reducing Fe^{3+}–*o*-phenanthroline, or alternatively oxygen might decrease the stability of the iron–sulfur group, resulting in the liberation of H_2S and secondarily affect protein conformation to make the iron more accessible to the reagents. However, the latter explanation is unfavorable as a predominant factor in the case of the *o*-phenanthroline reaction in view of the present results, particularly from experiments done in the presence of 4 M urea and 1 M KCl. It is of interest to note that the denaturation reaction of adrenodoxin by chaotropic agents is also stimulated by molecular oxygen (Kimura, 1968a). In this case, the changes in protein conformation of adrenodoxin by chaotropic agents results in a small alteration of the iron–sulfur group making it sensitive to molecular oxygen. There might be some basic similarities in terms of the oxygen effects between the chaotropic denaturation and *o*-phenanthroline reactions. On the other hand, the Tiron reaction which has no reductive step is not affected by 1 M KCl or by molecular oxygen.

The effects of 1 M KCl are at present difficult to understand. To be noted here is the fact that the effects are observed only in the case of the *o*-phenanthroline reaction but not in the case of the Tiron reaction. In relation to this, Nakamura (1970) in this laboratory found that in the presence of spinach ferredoxin, ferredoxin–NADP reductase produces superoxide anion in higher ionic strength media. This may support the idea that the effects of oxygen occur because of a radical reaction.

In this context, the previous implication that chemical approaches toward the valence state of iron in proteins is not reliable,[2] should somewhat be revised. In particular, the reactivity of Tiron would be reflecting the valence state of iron in the native oxidized protein.

The comparison of the reactivity of adrenodoxin with that of clostridial ferredoxin is of interest, although exact comparison is not possible because of different experimental conditions. The rates of reaction of adrenodoxin with *o*-phenanthroline and Tiron are much slower than those of ferredoxin. Therefore, it is concluded that the iron in adrenodoxin is less accessible to the iron chelators than it is with ferredoxin. From our experiments on reduced adrenodoxin, the faster rate observed in bacterial ferredoxin would not be due to the presence of Fe^{2+} ions in this oxidized protein. Rather, the difference can be ascribed to different protein conformations between helical adrenodoxin (Kimura *et al.*, 1969) and non-helical bacterial ferredoxin (Malkin and Rabinowitz, 1967).

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Mechanism of Action of 2,3-Diphosphoglycerate-Independent Phosphoglycerate Mutase*

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ABSTRACT: An improved method of purification of the diphosphoglycerate-(DPGA) independent phosphoglycerate mutase from wheat germ is described. By gel filtration the molecular weight was estimated to be 54,000. The reaction may involve (a) an intramolecular migration of phosphate, (b) an intermolecular transfer of phosphate from DPGA (present as contaminant or synthesized by the enzyme) to the substrates, (c) a phosphoenzyme mechanism, or (d) an intermolecular transfer of phosphate between two or more molecules of substrate. There was no induced transport with ^{14}C- or ^{32}P-labeled substrates and ^{32}P- and ^{14}C-labeled substrates at chemical equilibrium exchanged at equal rates. Mechanisms b and c were therefore excluded. A close adherence to Michaelis–Menten kinetics (K_m = 0.3 mM) with no sigmoid element and a lack of cotransport even at low concentration of substrates excluded mechanism d. The absence of binding of the substrates or phosphate by the enzyme in gel filtration studies with columns equilibrated with substrates and the lack of exchange between the substrates and DPGA provided additional evidence excluding these schemes. The enzyme, therefore, catalyzes an intramolecular transfer of phosphate (a). Since there was no counter transport at high substrate concentrations (20 mM) the rate constant for any isomerization of the free enzyme must probably be in excess of 1.0×10^6 sec^{-1}. Cyclic glyceric 2,3-phosphate was not a substrate, activator or inhibitor; exchange between the substrates and the ester could not be demonstrated; and the ester could not be isolated from the enzyme–substrate complex. The participation of the cyclic ester or a cyclic pentacovalent intermediate is therefore considered unlikely. The phosphatase activity of the enzyme involves the formation of an enzyme phosphate since exchange with the substrates of [^{14}C]glyceric acid but not [^{32}P]P_i was shown. The mechanism of the mutase may therefore involve the transient formation of an enzyme phosphate and a free glyceric acid molecule which remains bound to the enzyme until rephosphorylated: the enzyme may thus be related to the phosphatases. The marked difference from the DPGA-dependent enzymes suggests that these are two separate families of mutase enzymes.

The phosphoglycerate mutases from wheat and rice germ and from a number of other vegetable sources are 2,3-diphosphoglycerate (DPGA)[1] independent (Towne *et al.*, 1956; Ito and Grisolia, 1959; Grisolia and Joyce, 1959; Fernandez and Grisolia, 1960; Grisolia, 1968). In contrast, the activity of the phosphoglycerate mutases from muscle, yeast, and a number of other sources is very markedly stimulated by DPGA although there may be some small residual activity in the absence of this cofactor (Grisolia, 1968). In this paper the mechanism of the DPGA-dependent mutase of wheat germ is investigated.

The conversion of 2-phosphoglycerate (2-PGA) to 3-phosphoglycerate (3-PGA) by phosphoglycerate mutase may be represented by the steps

$$n(\text{2-PGA}) + E_1 \underset{k_{-1}}{\overset{k_{-1}}{\rightleftharpoons}} E(PGA)_n \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} + n(\text{3-PGA}) \quad (1)$$

$$E_1 \underset{k_{+3}}{\overset{k_{-3}}{\rightleftharpoons}} E_2 \quad (2)$$

where $E(PGA)_n$ represents the enzyme–substrate complex, and $E_1 \rightleftharpoons E_2$ an isomerization of the free enzyme. "*n*" indicates the number of substrate molecules which must combine with the enzyme before reaction can occur: it will be unity for most mechanisms unless the enzyme possesses more than one active center and there is cooperativity between the centers.

Possible mechanisms for the transfer of phosphate by wheat-germ phosphoglycerate mutase are summarized in Figure 1. Scheme A represents the direct intramolecular transfer of phosphate between two hydroxyl groups. The acid-catalyzed interconversion of 2-PGA and 3-PGA (Ballou and Fisher, 1954) probably proceeds in this manner with the intermediate formation of a cyclic 2,3-phosphoglycerate ester but the DPGA-dependent enzymes probably do not use this mech-

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[1] Abbreviations used are: 3-PGA, D-3-phosphoglyceric acid; 2-PGA, D-2-phosphoglyceric acid; DPGA, D-2,3-diphosphoglyceric acid; ADP, adenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate; NADH, reduced nicotinamide-adenine dinucleotide.