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Glycolic Acid Oxidase Formation in Greening Leaves [1, 2, 3]

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The amount of glycolic acid oxidase in green tissue is much greater on a protein nitrogen or weight basis than in tissue without chlorophyll (8, 10, 13, 15). The active enzyme cannot be isolated from roots and tubers (10, 13), but it can be detected in small amounts from etiolated tissues. During greening of the plant tissue in the light the activity of the oxidase increases immensely. Increased enzyme activity has been found in etiolated tissue kept in the dark upon feeding an excess of glycolate to the intact leaves. When glycolate was added to a cell-free extract from etiolated leaves, the enzyme activity increased greatly after 18 hours of incubation at 2 C (15).

An initial explanation for these phenomena was based upon substrate activation of the enzyme and the assumption that glycolate was not present in roots or etiolated tissue (15). For green plants it was known that large amounts of glycolate were produced by photosynthesis (11). However, the presence of some glycolate has since been reported in both roots and etiolated tissue (5, 6, 7). Thus a substrate activation hypothesis seems unsatisfactory unless the possibility of compartmentalization within the cell is invoked. In this paper we have reinvestigated the previous observations on the activation of glycolic acid oxidase. A substantial amount of proenzyme for glycolic acid oxidase has been found in etiolated plants, but in amounts insufficient to account for all the active enzyme in the corresponding green tissue. Since the cofactor for this enzyme is FMN (16), the level of FMN and FAD in etiolated green plants was also measured. Preliminary studies were made on conditions for holoenzyme formation.

Materials & Methods

Etiolated wheat *Triticum vulgare L,* var. Thatcher, was grown in sand with or without nutrient in a totally dark room at about 21 C for 9 to 10 days at which time the plants were about five inches tall. The leaves were ground in a cold mortar immediately

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after harvesting and the sap squeezed through cheese cloth and centrifuged at 200 × *g* for 5 minutes to remove debris. The cell free sap was adjusted to pH 8.3 with KOH when glycolate was to be the substrate or 7.3 when glyoxylate was used. Oxidation of glycolate was measured manometrically in the presence of a final concentration of 0.033 M phosphate buffer. Total nitrogen in the sap was determined by the semimicro Kjeldahl method. The data are expressed as Qo_2^N for the oxygen uptake during the period from 5 to 25 minutes after addition of the substrate. Ammonium sulfate fractionation of the enzyme was performed as previously described (4). The spraying of intact plants in the dark with substrates has previously been mentioned (15). The flavin content was determined fluorometrically (2, 3) with an electronic fluorometer, model 12B of Coleman Instruments, Inc.

Results & Discussion

► Activation of Enzyme by FMN: In the absence of added FMN an active glycolic acid oxidase was present in the sap from green Thatcher wheat leaves, but in the sap from the etiolated leaves only a small amount of activity was present (table I, II, & III). These results confirm previous reports of this phenomenon as discussed in the introduction. No active enzyme in the extract from roots of green peas or wheat seedlings was detectable in the presence or absence of added FMN. Glycolic acid oxidase has also been reported to be absent from the sheaths of gramineous plants, although adjacent green leaves showed normal enzymatic activity (8). Sap from the sheaths and leaves of green or etiolated wheat seedlings was prepared after careful visual separation of the sheaths. In this sap without added FMN about four per cent as much enzyme activity on a Qo_2^N basis was found as in the leaves of the same plants (table I, lines 1 & 3).

Table I

Glycolic Acid Oxidase in Leaves & Sheaths of Thatcher Wheat

Kind of tissue	FMN Final conc	Qo_2^N
Green leaves	0	55
	1×10^{-4} M	108
Sheaths from green plants	0	2
	1×10^{-4} M	89
Etiolated leaves	0	7
	1×10^{-4} M	55
Sheaths from etiolated plants	0	6
	1×10^{-4} M	17

Enzyme activation occurred upon the addition of FMN to cell free saps from both green and etiolated wheat leaves or wheat sheaths (table I), but not from roots. In order to obtain the maximal enzymatic activity from saps of plant tissue, a final added concentration of 10^{-4} M FMN was necessary (table II). The data indicate that proenzyme was present in etiolated leaves and in green sheaths, though little holoenzyme could be isolated from these tissues. The maximum enzyme activity from extracts of both green and etiolated leaves was obtained between 10^{-4} and 10^{-3} M FMN. Added FMN increased the oxidase activity in extracts from etiolated leaves about tenfold, whereas the enzyme activity in extracts from green leaves was increased about 2.5-fold. Nevertheless, green tissue always contained on the basis of Qo_2^N about two to three times more total enzyme than etiolated leaves.

Table II

FMN Activation of Glycolic Acid Oxidase in Sap from Green & Etiolated Wheat Leaves

Final FMN conc	Glycolate substrate		Glyoxylate substrate	
	Green	Etiolated	Green	Etiolated
Molar	Qo_2^N	Qo_2^N	Qo_2^N	Qo_2^N
0	40	4	3	0
1×10^{-7}	39	7	2	1
1×10^{-6}	55	12	2	1
1×10^{-5}	95	30	6	1
1×10^{-4}	105	42	27	4
1×10^{-3}	107	41	27	7
1×10^{-2}	95	37	30	5

The rate of oxidation of glyoxylate by the sap from green or etiolated leaves was slow, and the addition of FMN also stimulated this rate. This oxidation is also catalyzed by glycolic acid oxidase (12), but the K_M for glyoxylate is less than for glycolate.

► Effect of Light Plus FMN on Enzyme Activity: As mentioned in the introduction, glycolic acid oxidase increased in vivo manifold during the greening of etiolated plants. Since proenzyme was present in the extract from etiolated leaves, the increase in holoenzyme in vivo during greening might be the result of an association of FMN with a proenzyme. However, even in the presence of excess FMN the amount of demonstratable total enzyme in etiolated sap was only one-third to one-half that present in a comparable green leaf (table II). In a further investigation of this discrepancy plants of the same age were exposed to light for varying lengths of time (table III) and then assayed for the holoenzyme (column 2) and for the total of proenzyme plus holoenzyme which could be revealed by the addition of 10^{-4} M FMN (column 3). Exposure to light stimulated the formation of active enzyme as well as the formation of a large amount of proenzyme. In all cases there was maintained a five- to sixfold excess of the proenzyme over the active enzyme as indicated by the ratios in column 4. This ratio remained nearly constant after the initial exposure to light. It is thought that the excess of proenzyme was not the result of cleavage of the holoenzyme during grinding of the leaves, because the FMN was not readily re-

Table III

Effect of Light During Growth & Addition of FMN to Sap on Activity of Glycolic Acid Oxidase*

Exposure to light	Additions during assay		
	Glycolate	Glycolate + FMN	Ratio Glycolate + FMN/ glycolate
	Qo_2^N	Qo_2^N	
0	5.3	47.5	9.0
3 hr	9.8	50.6	5.2
6 hr	10.9	70.2	6.4
12 hr	14.0	92.7	6.6
2 days	19.2	121.8	6.3
3 days	24.6	133.2	5.4
4 days	29.6	158.1	5.3

* Before exposure to light the plants had been grown in total darkness. Age of all plants at harvest was 10 days, regardless of prior period of exposure to light. The manometric assays were run either with glycolate or with glycolate plus 1×10^{-4} M final FMN concentration.

moved from the isolated holoenzyme, (4, 14, 16). Thus, in the light and in vivo two processes occurred. Initially, within three hours, there was a doubling of active enzyme without significant change in the total sum of proenzyme plus holoenzyme. In continuing light there was a build up of the total enzyme protein which would be the result of new protein synthesis.

► Flavin Content of Wheat Leaves: In the above section the constant excess of proenzyme in wheat leaves suggested that a controlling factor for glycolic acid oxidase activity might be the availability of FMN. Consequently, these Thatcher wheat leaves were assayed for FMN, FAD, and riboflavin (table IV). The total flavin content of the leaves did not differ significantly between green or etiolated tissue, but the distribution between FAD and FMN was strikingly different in these two types of leaves. The FAD content of etiolated leaves was twice that in green leaves. On the other hand, the FMN content of green leaves was twice that of etiolated leaves. It is possible that during the greening of the leaves FAD was partially hydrolyzed to FMN and AMP by nucleotide pyrophosphatase and thus the total flavin content would not have changed. The following experiments indicated that such hydrolysis might have occurred in vivo. When 1.6×10^{-7} mole of FAD was incubated for 1 hour with 0.5 ml of cell free sap from etiolated wheat and 2.5 ml of 0.03 M phosphate buffer at pH 8.3, only 1 % of the flavin was recovered as FAD, 90 % was recovered as riboflavin and 9 % as FMN. Although FAD is not the coenzyme for glycolic acid oxidase (16), addition of FAD to etiolated sap activated the enzyme probably because the FAD was hydrolyzed to FMN.

In spite of the important role of FMN in respiration, the FMN content of leaves is remarkedly low. Our values for Thatcher wheat were somewhat higher than those which Asomaning and Galston have reported for some other grains (1), but in the range reported by Kondo for bean leaves (9). Though Asomaning and Galston did not record the FAD content, their data showed a doubling of FMN content upon exposure of the etiolated seedling to light (table V of reference 1).

Table IV

Flavins in Thatcher Wheat Leaves*

Flavin	γ/g Dry wt		Calculated molarity	
	Green	Etiolated	Green	Etiolated
FAD	46	108	4×10^{-9}	6×10^{-9}
FMN	93	53	1×10^{-8}	5×10^{-9}
Riboflavin	2	5	4×10^{-10}	9×10^{-10}
Total	141	166	...	...

* Molarity was calculated on the basis of γ of flavin per gram of dry weight and the volume of sap which was obtained from an equivalent quantity of fresh tissue. All plants were assayed about ten days after planting the seed.

Table V

Qo_2^N of Glycolic Acid Oxidase in Cell-Free Wheat Sap After 18 Hours Preincubation*

Preincubated with	Additions during assay	Etiolated	Green
Water	Glycolate	10	68
Water	Glycolate + FMN	53	106
Glycolate	Glycolate	58	104
Glycolate	Glycolate + FMN	69	106
Lactate	Glycolate	37	113
Lactate	Glycolate + FMN	86	146
α-Hydroxybutyrate	Glycolate	35	114
α-Hydroxybutyrate	Glycolate + FMN	92	150

* Cell free sap was incubated for 18 hours at 2 C in 0.05 M phosphate buffer at pH 8.3. The final concentration of substrate during incubation was 0.008 M which was obtained by adding the appropriate volume of 0.1 M solution. Afterwards during the Warburg assay 0.024 M glycolate was added, and, when indicated, FMN at 1×10^{-4} M final concentration.

The increased FMN content of the green leaves was consistent with the increase in activity of the FMN requiring glycolic acid oxidase. The results do not, however, fully explain the difference in activity of glycolic acid oxidase in etiolated compared to green tissue. The FMN content of etiolated tissue was about one-half that of green tissue, yet the active enzyme in etiolated wheat was very low even though the proenzyme and coenzyme were present. The estimated molarity of FMN in these tissues was between 10^{-8} and 10^{-9} (table IV). In vitro it was necessary to increase the FMN molarity to the range of 10^{-6} to 10^{-5} before activation of the proenzyme was possible and 10^{-4} M FMN was necessary to fully activate the enzyme. Thus a change of FMN concentration from 5×10^{-9} M in etiolated tissue to 1×10^{-8} M in green tissue might not by itself suffice to activate the enzyme. The physiological optimal

level of FMN necessary to activate the enzyme in vivo cannot be determined from our data. However, the order of magnitude of 10^{-8} M FMN in green leaves containing an active glycolic acid oxidase is much different than the 10^{-4} M FMN needed in vitro to activate the proenzyme. This comparison suggests that the in vivo formation of the holoenzyme is in addition an active process and perhaps enzymatic.

► Activation of Glycolic Acid Oxidase in Vitro by Prolonged Substrate Incubation: Glycolic acid oxidase can be activated in a cell free homogenate from etiolated wheat leaves by incubating the sap in the cold with glycolate for 18 hours (table V, line 3) but not with water and buffer (line 1). After grinding the leaves, the preincubation with substrates, as indicated in column 1, was started in about 15 minutes, which was the time necessary for adjusting the pH and centrifuging. It should be emphasized that this experiment was conducted without the addition of FMN. However 10^{-4} M FMN was added to half the samples during the assays to determine the maximum enzyme activity to be expected from the proenzyme present (line 2). Excess glycolate was used in all manometric assays. The maximum enzyme activity found with 10^{-4} M FMN occurred immediately upon addition of the FMN and was the same before and after preincubation. Activation of the enzyme by glycolate alone at 30 C in the manometric vessels did not occur in the length of time required for the assay. The phenomenon was best demonstrated by preincubation at 2 C to keep heat inactivation to a minimum amount, but it was necessary to run the final assay at 30 C. The enzyme was also activated when cell free sap was incubated with lactate or α-hydroxybutyrate which are substrates for the active enzyme (4). Preincubation of the sap with glyoxylate or H_2O_2 did not activate the enzyme.

Increased enzyme activity in vitro by prolonged preincubation with its substrates also occurred in sap from green leaves (table V, column 4). After incubation with glycolate the final enzyme activity without added FMN was nearly equal to the maximal activity obtained with addition of 10^{-4} M FMN.

Activation of the proenzyme of glycolic acid oxidase in an ammonium sulfate fraction was not achieved by incubation with its substrate and 10^{-8} M FMN. Protein fractions were collected from etiolated or green wheat sap between 18 to 28 g of $(NH_4)_2SO_4$ per 100 ml of solution, because this fraction would contain the active glycolic acid oxidase of green leaves (4). After dissolving the protein precipitate in water and dialyzing for 4 to 8 hours to remove $(NH_4)_2SO_4$, the proenzyme was incubated with glycolate and 10^{-8} M FMN. No activation occurred. Full activation of the enzyme in the dialyzed ammonium sulfate fraction occurred upon addition of 10^{-4} M FMN, and the amount of active enzyme was nearly equal to the proenzyme originally present in the sap from the etiolated leaves.

► Immediate Activation of Glycolic Acid Oxidase in Vitro by Substrate: For the experiments in table V, no activation of the enzyme occurred when the substrate was added at the beginning of the manometric assay. In these cases substrate had been added from the side arm of the flask about 40 minutes after grinding the leaves. This length of time was necessary to prepare the sap and start the manometric experiments. However, if the glycolate were added to the plant sap immediately upon squeezing the sap from the crushed leaves or if the etiolated leaves were ground with a glycolate solution, an active glycolic acid oxidase was present in the sap from the etiolated leaves as soon as the assay could be run and no 18-hour preincubation was necessary (table VI & VII). This rapid activation also occurred without the addition of FMN. The glycolic acid oxidase activity found in the sap from green leaves was increased also several fold by addition of substrate im-

Table VI

Activity (Qo_2^N) of Glycolic Acid Oxidase in Extracts from Etiolated or Green Wheat Leaves When Prepared With Glycolate*

Grinding solution	Additions during assay	Etiolated	Green
Water	Glycolate	6	42
Water	Glycolate + FMN	87	165
Glycolate	Glycolate	69	144
Glycolate	Glycolate + FMN	111	162

* To all saps were added during grinding of the leaves sufficient 1 M phosphate buffer to give a final concentration of 0.05 M at pH 8.3, and glycolate as indicated. All incubation mixtures with glycolate were prepared by adding sufficient 0.1 M glycolate to give a final concentration of 0.024 M. All final FMN concentrations were 1×10^{-4} M. The addition of glycolate during the manometric assay amounted to 0.5 ml of 0.05 M to assure an excess of substrate. Manometric measurements were begun within 30 minutes after grinding the leaves.

Table VII

Activity (Qo_2^N) of Glycolic Acid Oxidase in Etiolated Leaf Sap to Which Glycolate & FMN Were Added*

Molarity during incubation		Molarity FMN added to assay	Time and temp. of incubation		
Glycolate	FMN		30 min** 30 C	4 hr 2 C	18 hr 2 C
0	0	0	8	6	2
0	10^{-7}	0	9	6	4
0	10^{-6}	0	34	24	11
0	10^{-5}	0	80	70	53
0	0	10^{-4}	82	62	28
0.008	0	0	53	52	47
0.008	10^{-7}	0	48	49	46
0.008	10^{-6}	0	67	57	47
0.008	10^{-5}	0	79	79	67

* Immediately upon grinding the leaves, 1 M phosphate was added to make a final concentration of 0.05 M at pH 8.3; 0.1 M glycolate was added where indicated to a final concentration of 0.008 M, and FMN was added to give the indicated final molarity. At the beginning of all manometric assays an additional 24 μmoles of glycolate were added.

** The shortest incubation period at 30 C for 30 minutes was dictated by the time and temperature needed for grinding the leaves and preparing for the manometric assay.

mediately upon grinding the leaves. The activity obtained by quick addition of glycolate to the sap was more than half of the total enzyme which could be detected by addition of excess (10^{-4} M) FMN. Thus there were two types of substrate activation: A, very rapid activation if the substrate were added as the leaves were homogenized and B, slow activation requiring many hours if addition of the substrate was delayed for 20 to 40 minutes after grinding the leaves. The same level of enzyme activity could be obtained by either method.

The presence of the substrate also prevented destruction of the proenzyme during prolonged incubation periods. When the sap was incubated for 18 hours with only buffer the Qo_2^N dropped from 82 to 28 as measured afterward with excess FMN (table VII, line 5). When the enzyme was incubated with its substrate for 18 hours, the decrease in final activity was less. The addition of low concentrations of FMN (10^{-7} to 10^{-6} M) along with glycolate did not significantly increase the enzyme activity during short or long periods of incubation above that obtained by glycolate alone.

► Substrate Activation in Vivo: It has previously been demonstrated that spraying glycolate on etiolated plants while they remained in darkness, resulted, within 24 hours, in the presence of more active enzyme in the sap from these leaves (15). We have now observed that about a twofold increase in activity also occurs with lactate, α-hydroxybutyrate or glyoxylate, all substrates for the enzyme (table VIII). Little activation occurred with acetate, which is not a substrate for the enzyme. The total proenzyme plus holoenzyme in the sap from these treated plants were determined after the addition of 10^{-4} M FMN, and all plants contained nearly the same amount before or after treatment. The substrate treatment in vivo appeared to have resulted in partial conversion of the proenzyme to holoenzyme, but this treatment did not increase the total proenzyme plus holoenzyme content. The latter process, which was demonstrated in table III, occurred in the light in vivo.

Table VIII

Activity (Qo_2^N) of Glycolic Acid Oxidase After in vivo Treatment with Substrates*

Spray treatment	Assay conditions	
	Glycolate	Glycolate + FMN
Water	10	89
Glycolate	38	104
Lactate	27	104
α-Hydroxybutyrate	30	116
Glyoxylate	25	97
Acetate	14	

* Each flat of 7 day old etiolated Thatcher wheat seedlings was sprayed with 10 μmoles of a designated compound in 100 ml of water. Plants were maintained in total darkness during spraying and until harvest 24 hours later. During the manometric assay 10 μmoles of glycolate were added and where indicated a 1×10^{-4} M final FMN concentration was also used.

Discussion

One of the physiological questions for which these experiments were designed was whether etiolated leaves contain glycolic acid oxidase or whether this enzyme was characteristic only of green tissue. Although only low enzyme activity in sap from etiolated leaves was observed by the usual manometric procedure in which the substrate was added from the side arm, much activity was found by four different means. A, Addition of 10^{-4} M FMN quickly and fully activated an abundant proenzyme in the sap from etiolated leaves. This phenomenon is characteristic of activation of an apoenzyme by its cofactor. B, By grinding the leaves with glycolate or by adding glycolate to the leaf sap as soon as it was obtained from the crushed leaves, an active enzyme was obtained without addition of FMN. This phenomenon is characteristic of substrate protection of an enzyme. C, If the sap were incubated with buffer but no glycolate for 15 to 30 minutes after it was obtained from the leaves, then subsequent incubation with glycolate for long periods of about 18 hours at 2 C was required before the enzyme activity was fully restored. D, If the leaves were sprayed with substrate 24 hours before harvest more active enzyme was present in the sap. This phenomenon is characteristic of substrate activation. In most cases the total enzyme which could be activated in sap from etiolated leaves was two- or threefold less than the total enzyme present in the sap from a corresponding green plant. However, the green plant also contained a major part of its glycolic acid oxidase in an inactive form which had to be activated by one of the above procedures.

Although the mechanism of glycolic acid oxidase activation during greening of the plant is still not known, the results suggest that considerable proenzyme was present in the etiolated plant. During greening some change resulted in partial activation of the enzyme. Two such changes might be increases in FMN or glycolate concentrations at specific sites. Another interpretation of these results is that in the etiolated leaves glycolic acid oxidase was in an active form and was inactivated during preparation of the sap. Destruction might consist of cleavage of the holoenzyme into a stable proenzyme and FMN, unless the system was protected by its substrate. Further the FMN would be destroyed by enzymatic hydrolysis during the time necessary to prepare for the manometric assay. Quantitative analysis of FAD added to etiolated sap showed that it was rapidly hydrolyzed to FMN and then to riboflavin. Glycolic acid oxidase activity could be restored at any time by addition of excess FMN. Some of the data can not be explained by this theory concerning substrate protection of an otherwise active enzyme from etiolated leaves. Holoenzyme was obtained from green leaves and it was stable for long periods of time in the sap. Some active enzyme from etiolated leaves was stable in sap. The hypothesis does not explain why the enzyme without substrate was inactive in etiolated sap after 15 minutes, but subse-

quently its activity could be restored slowly by incubation with substrate. During the incubation time without substrate all free FMN should have been hydrolyzed, yet no additional FMN was required during the 18 hour incubation period needed to reactivate the enzyme. In fact addition of physiological amounts of 10^{-8} to 10^{-6} M FMN by itself for a short or long incubation period had no influence upon the activity of the enzyme.

The enzyme was active in etiolated plant sap which contained about 10^{-9} M FMN providing the substrate had been added to the freshly prepared sap. Without immediate addition of substrate 10^{-4} M FMN was needed for activation. The ammonium sulfate fraction which contained the proenzyme was not activated by the substrate with or without 10^{-8} to 10^{-6} M FMN. However 10^{-4} M FMN fully activated the ammonium sulfate precipitated proenzyme. The ammonium sulfate precipitation of holoenzyme did not remove FMN. Thus, the ammonium sulfate precipitation of the proenzyme either altered the fine structure of the protein so that substrate and low concentrations of FMN could not reactivate it or an activating enzyme was lost.

The literature indicates that a large amount of the terminal glycolic acid oxidase is normally found in green plant tissue. If those assays had been performed with excess FMN or the use of glycolate during extraction of the tissue, values two- to threefold higher may have been found. Because the potential activity of this system in vivo is so great some control of it must exist. Interactions between the proenzyme, FMN and substrate may function in some manner for this control.

Summary

The glycolic acid oxidase activity in the sap from etiolated Thatcher wheat leaves is 10 % or less of the activity from the corresponding green leaves. The activity of the enzyme in extracts from etiolated leaves may be increased the same five- to tenfold by addition of 10^{-4} M FMN, by grinding the leaves with glycolate, or by incubation of the inactive sap for 18 hours at 2 C with a substrate for the enzyme. The enzyme activity is also increased by spraying etiolated leaves 24 hours prior to harvest with one of the substrates. After one ammonium sulfate fractionation of the proenzyme, the in vitro activation could only be accomplished by 10^{-4} M FMN.

Although a very active glycolic acid oxidase was always found in extracts from green leaves, the activity may also be increased two- to fivefold by addition of 10^{-4} M FMN or by grinding the green leaves with glycolate. The total enzyme which can be revealed by excess FMN from green leaves was about two- to threefold greater on a Qo_2^N basis than the total detectable enzyme from the etiolated leaves.

During greening of the etiolated leaves there was no change in the total flavin content. However the FAD concentration of the etiolated Thatcher wheat leaf was about double the FMN content while the FMN content of the green leaf was about double the FAD concentration. The total FMN molarity based upon volume of leaf extract was between 5×10^{-9} to 1×10^{-8} M, while 10^{-4} M FMN was needed to activate the enzyme in vitro.

These results suggest that either considerable amount of proenzyme was present in the etiolated leaves or that a labile form of the holoenzyme was present and was destroyed during grinding of the leaves. However neither interpretation fully explains all of the results.

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