Effect of Adenosine 5′-Triphosphate and Adenosine 5′-Diphosphate on the Oxidation of Cytochrome *c* by Cytochrome *c* Oxidase†

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ABSTRACT: Adenosine 5′-triphosphate (ATP), adenosine 5′-diphosphate (ADP), and inorganic pyrophosphate partially inhibit the oxidation of exogenous cytochrome *c* by cytochrome *c* oxidase of submitochondrial particles (with or without detergent treatment) or by a purified preparation when it is assayed polarographically in buffers of nonbinding ions at pH 7.8. ATP is somewhat more inhibitory than ADP. The inhibition is never greater than 50%, and it is always less when an equal concentration of Mg^{2+} ions is present or when the assays are run at pH 6. In contrast, the effect of ATP, ADP, and pyrophosphate on oxidase assays run spectrophotometrically is a similar slight stimulation of the oxidase of submitochondrial particles treated with deoxycholate and little or no effect on purified oxidase. The reaction of the oxidase of submitochondrial particles with the endogenous cytochrome *c* is stimulated by the nucleotides, as is the reduced nicotinamide adenine dinucleotide (NADH) oxidase activity. The observations can be explained by binding of ATP, ADP, or pyrophosphate to cytochrome *c* so that the formation of an especially reactive combination of cytochrome *c* and cytochrome oxidase previously postulated [Smith, L., Davies, H. C., & Nava, M. E. (1979) *Biochemistry 18*, 3140] is prevented. The data give no evidence that respiration via cytochrome *c* oxidase is regulated physiologically by direct effects of ATP or ADP on its activity.

Recent studies on direct effects of ATP and ADP on cytochrome *c* oxidase activity lead to the suggestion that these effects play a regulatory role on the overall rate of O_2 uptake by the respiratory chain system. From polarographic assays Ferguson-Miller et al. (1976) reported that the "apparent K_M" for a high-affinity binding site for cytochrome *c* to the oxidase of beef heart mitochondrial membranes was increased to a greater extent by ATP than by ADP. Roberts & Hess (1977) studied effects of the nucleotides on yeast cytochrome *c* oxidase assayed spectrophotometrically (with high concentrations of cytochrome *c*) and polarographically (with both low and high concentrations of cytochrome *c*). ATP was found to inhibit oxidase bound to membranes from promitochondria in buffers of both high and low ionic strength, but the purified oxidase was inhibited only under conditions of low ionic strength. ATP abolished the activation of a lipid-deficient purified oxidase by the addition of promitochondrial membranes, leading to the suggestion that ATP prevents the attraction or binding of cytochrome *c* to the membranes; ADP had a lesser effect.

We have made in-depth studies of the two usual methods for observing the kinetics of reaction of cytochrome *c* with cytochrome oxidase: the spectrophotometric (oxidation of soluble ferrocytochrome *c*) and the polarographic (O_2 uptake with cytochrome *c*, TMPD,[1] and ascorbate) (Smith et al., 1979a,b). Our data indicate that with the two methods different rate-limiting steps may be involved. Under some experimental conditions the turnover rates measured polarographically are much higher than could be expected from the rates of oxidation of cytochrome *c* measured spectrophotometrically. These observations and others suggested the formation of an especially reactive combination of cytochrome *c* and cytochrome oxidase under some conditions.

In Tris[1]–cacodylate buffer, pH 7.8 (optimal conditions for polarographic assays), ATP, ADP, or pyrophosphate, with or without Mg^{2+} ions, partially inhibits the oxidase activity measured polarographically at low concentrations of cytochrome *c* but stimulates the activity measured spectrophotometrically. Thus, the turnover rates measured by the two methods in the presence of ATP no longer show the large discrepancy seen in its absence, and the evidence for the highly reactive combination of cytochrome *c* with cytochrome oxidase is no longer apparent. This suggests that substances such as ATP that bind to cytochrome *c* can inhibit the formation of such a compound. However, our data give no evidence that the effects of ATP and ADP have any significance for the regulation of O_2 uptake by direct effects on cytochrome *c* oxidase. Preliminary presentation of some of these data has been made (Davies et al., 1979).

Materials and Methods

Preparations. Submitochondrial particles (SMP) were isolated from beef heart mitochondria by the method of Lee & Ernster (1967). With some preparations the mitochondria were made deficient in cytochrome *c* before preparation of the SMP, as described previously (Smith et al., 1979b); these were then treated with DOC[1] to rupture the membrane vesicles and to expose the oxidase maximally for reaction with cytochrome *c* (Smith & Camerino, 1963). Treatment with detergent also seemed to remove remaining endogenous cytochrome *c*.

Purified cytochrome aa_3 was prepared by the method of Hartzell & Beinert (1974). The content of cytochrome aa_3 in the different preparations was measured according to Vanneste (1966).

Cytochrome *c* was isolated from beef heart following the directions of Margoliash & Walasek (1967) and was further purified by isoelectric focusing (Smith, 1978). Its concen-

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[1] Abbreviations used: SMP, submitochondrial particles; TMPD, *N,N,N′,N′*-tetramethylphenylenediamine; DOC, sodium deoxycholate; Tris, tris(hydroxymethyl)aminomethane; Hepes, *N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulfonic acid; ATP, adenosine 5′-triphosphate; ADP, adenosine 5′-diphosphate; NADH, reduced nicotinamide adenine dinucleotide.

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tration in solution was calculated from the absorbance at 550 nm of the reduced form (Margoliash & Frohwirt, 1959).

Assay Methods. Cytochrome *c* oxidase was assayed polarographically with a Clark O_2 electrode in a temperature-controlled vessel of 2.6 mL in the presence of TMPD and ascorbate (Ferguson-Miller et al., 1976; Smith et al., 1979b). When the reaction with the endogenous cytochrome *c* of SMP was measured, only TMPD and ascorbate were added; then the additional O_2 uptake due to reaction with added exogenous cytochrome *c* was followed. There was little or no endogenous cytochrome *c* in the cytochrome *c* deficient SMP treated with DOC or in the purified cytochrome aa_3, and only rates with exogenous cytochrome *c* were assayed. NADH oxidase activity of SMP was measured after addition of 1 mM NADH. The experimental conditions are found in the legends to the figures. The rates are expressed as micromolar O_2 uptake per second.

Oxidase activity was measured spectrophotometrically with an Aminco DW2a spectrophotometer at 418 or 550 nm in the split beam mode following the method of Smith & Conrad (1956); first-order rate constants were calculated. Velocities of reaction were obtained from the product of the rate constants and of the concentrations of ferrocytochrome *c*. These, divided by four, gave the equivalent rates of O_2 uptake.

Chemicals. ATP (disodium salt) was obtained from Sigma Chemical Co. or from Boehringer-Mannheim and neutralized to pH 6.5 with Tris base. ADP (trilithium salt) was a P-L Biochemicals product; a solution in water gave a pH of 7. (Some samples of Sigma ADP contained substances which yielded KCN-insensitive O_2 uptake in the presence of ascorbate.) $MgCl_2$ was from Merck (reagent grade). TMPD, NADH, and sodium ascorbate were also obtained from Sigma Chemical Co. The sodium ascorbate was recrystallized at least twice from 70% ethanol.

Results

The effects of ATP, ADP, and pyrophosphate, with and without an equal concentration of $MgCl_2$, on the polarographically measured oxidase activity are documented in Figure 1A–C. It should be emphasized that the effects differed somewhat depending upon the type of preparation used and were always less when Mg^{2+} ions were present. Figure 1A is a plot of the activity of purified cytochrome aa_3 assayed under the optimal conditions for the polarographic method, with and without 3 mM ATP or ADP at different concentrations of cytochrome *c*. Both nucleotides are inhibitory at concentrations of cytochrome *c* between 0.05 and 0.5 μM and are slightly stimulatory at higher concentrations, ADP being somewhat less effective than ATP. The inhibitory effects on SMP treated with DOC were greater than on purified cytochrome aa_3. With 0.1 μM cytochrome *c*, where the inhibitory effect was greatest, addition of increasing concentrations of ATP to the oxidase of SMP treated with DOC produced increasing inhibition up to a concentration of 5 mM, where the inhibitory effect leveled off (Figure 1B). ADP was less inhibitory than ATP, and pyrophosphate was intermediate between the two. The plots of Figure 1C, also obtained from experiments with SMP treated with DOC, show that the inhibitory effect of ATP is decreased when an equal concentration of $MgCl_2$ is present. All together, the polarographic measurements with detergent-treated SMP or with purified cytochrome aa_3 show that ATP, ADP, or pyrophosphate, with or without Mg^{2+} ions, can partially inhibit the reaction of exogenous cytochrome *c* with the oxidase, but the maximal inhibition is around 50% with 5 mM ATP, which would be a rather high cellular concentration. The inhibition is less with the Mg salts, in which form ATP and ADP exist predominantly within the cells.

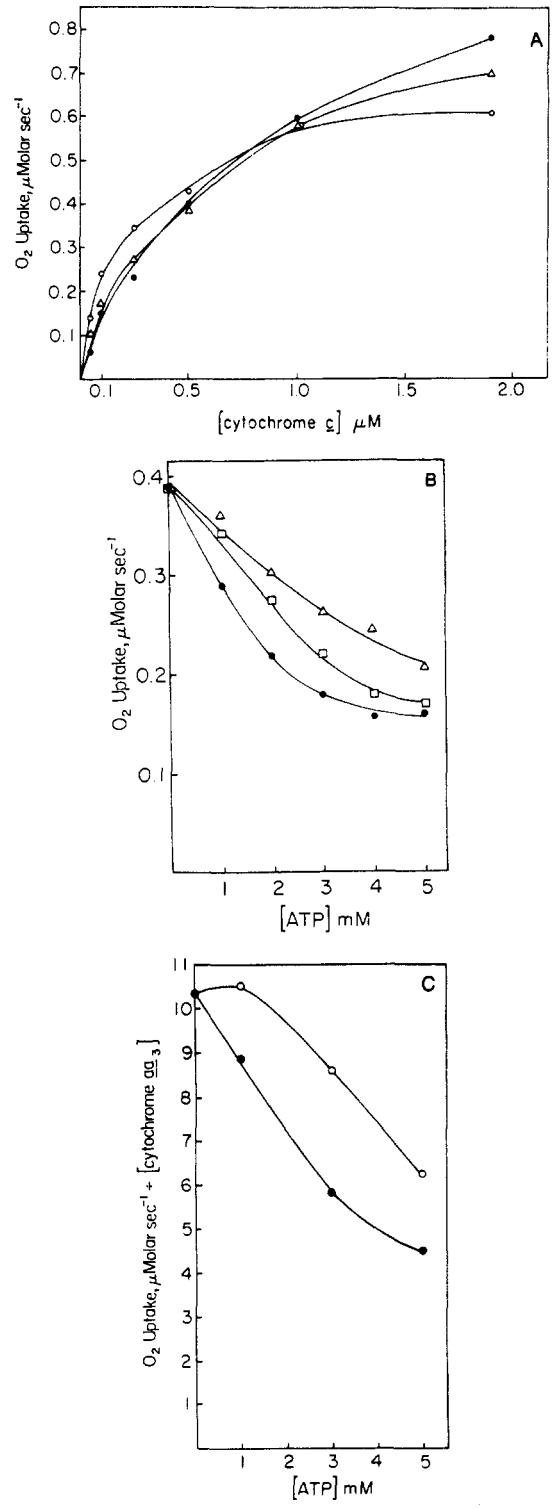

FIGURE 1: Polarographic oxidase assays in 0.025 M Tris–cacodylate buffer, pH 7.8, with 0.75 mM TMPD plus 10 mM ascorbate. (A) Assay mixtures contained 0.112 μM purified cytochrome aa_3: (○) control; (●) 3 mM ATP; (△) 3 mM ADP. (B) Effect of ATP, ADP, and pyrophosphate on oxidase activity of SMP treated with DOC containing 0.032 μM cytochrome aa_3 with 0.1 μM cytochrome *c*: (●) ATP; (△) ADP; (□) pyrophosphate. (C) Effect of ATP ± $MgCl_2$ on the oxidase activity of SMP treated with DOC. Assays contained 0.1 μM cytochrome *c* and SMP containing 0.032 μM cytochrome aa_3: (●) no Mg^{2+}; (○) +Mg^{2+}.

ATP and ADP were considerably less inhibitory when the polarographic assays were run at pH 6 (data not shown).

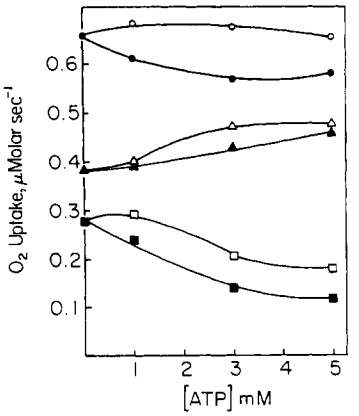

FIGURE 2: Effect of ATP ± $MgCl_2$ on the oxidase activity of SMP with endogenous and exogenous cytochrome *c*, measured polarographically. Assays were run in 0.025 M Hepes buffer, pH 7.8, with 0.75 mM TMPD plus 10 mM ascorbate and SMP containing 0.057 μM cytochrome aa_3 with and without 0.1 μM cytochrome *c*: (■) exogenous cytochrome *c*; (□) exogenous cytochrome *c* plus $MgCl_2$; (▲) endogenous cytochrome *c*; (△) endogenous cytochrome *c* plus $MgCl_2$; (●) endogenous plus exogenous; (○) endogenous plus exogenous plus $MgCl_2$.

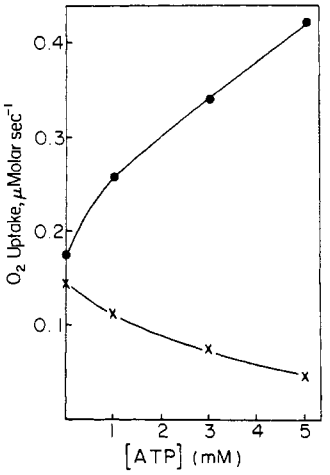

FIGURE 3: Effect of ATP on NADH oxidase and on NADH oxidase plus cytochrome *c*. Assays were run in 0.025 M Hepes buffer, pH 7.8, with 1 mM NADH and SMP (partially deficient in cytochrome *c*) containing 0.088 μM cytochrome aa_3, without and with 0.1 μM cytochrome *c*: (●) NADH oxidase; (×) O_2 uptake on addition of cytochrome *c*.

The inhibitory effects of ATP, with and without $MgCl_2$, were also incomplete on the oxidase of SMP (cytochrome *c* sufficient) not treated with detergent (Figure 2). Here there was a small stimulatory effect on the reaction with endogenous cytochrome *c* and an inhibitory effect on the reaction with exogenous 0.1 μM cytochrome *c*, so that the total O_2 uptake (endogenous plus exogenous) hardly changed. Similarly, the NADH oxidase of these preparations with the endogenous cytochrome *c* was stimulated by ATP, while the increase in O_2 uptake on addition of exogenous cytochrome *c* was inhibited (Figure 3).

The inhibitory effects of ATP and ADP seen in Tris–cacodylate or Hepes buffers were additive (data not shown). Also the inhibitory effects were somewhat greater if the ATP was added to the reaction mixture after the cytochrome *c*, rather than before the cytochrome *c*, but the difference was not very great (around 15%).

The addition of ATP, ADP, or pyrophosphate to the oxidase reaction measured spectrophotometrically resulted in very different effects from those seen with the polarographic assays. ATP and ADP (3 mM) were about equally stimulatory to the

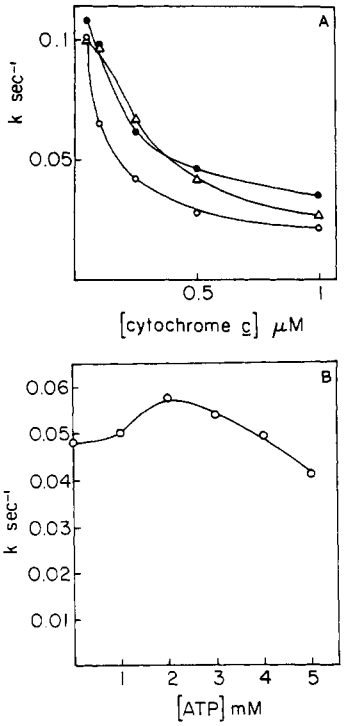

FIGURE 4: (A) Effect of 3 mM ATP or ADP on the oxidase activity of SMP treated with DOC (containing 1 nM cytochrome aa_3) assayed spectrophotometrically in 0.025 M Tris–cacodylate, pH 7.8: (○) control; (●) +ATP; (△) +ADP. (B) Effect of ATP concentration on the activity of purified cytochrome aa_3 (containing 0.56 nM cytochrome aa_3) measured spectrophotometrically in Tris–cacodylate buffer (0.025 M), pH 7.8, with 0.1 μM cytochrome *c*.

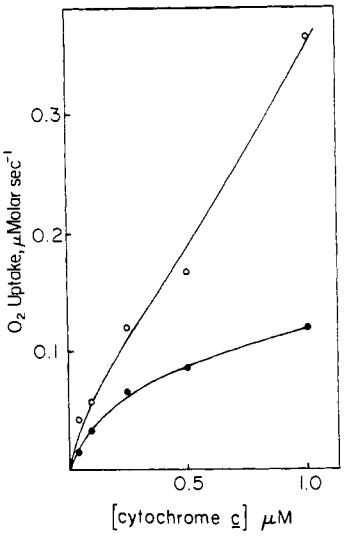

FIGURE 5: Comparison of oxidase activity of SMP treated with DOC measured polarographically and spectrophotometrically in the presence of 3 mM ATP. Assays were run in 0.025 M Tris–cacodylate buffer, pH 7.8, with 0.75 mM TMPD and 10 mM ascorbate in the polarographic assays: (○) O_2 uptake rates with SMP containing 0.0128 μM cytochrome aa_3; (●) corresponding rates calculated from spectrophotometrically measured rates.

oxidase of SMP treated with DOC with cytochrome *c* concentrations between 0.1 and 1 μM (Figure 4A). However, neither had much effect on the activity of purified cytochrome oxidase oxidizing 0.1 μM ferrocytochrome *c* (Figure 4B).

When SMP treated with DOC were assayed simultaneously by both polarographic and spectrophotometric methods in Tris–cacodylate buffer, pH 7.8, with 3 mM ATP, the data of Figure 5 were obtained. The O_2 uptake rates measured polarographically with the lowest concentrations of cytochrome

c (0.05–0.2 μM) were not very different from those calculated from the spectrophotometrically measured rates of oxidation of cytochrome *c* (usually less than double); at these low concentrations the so-called "high-affinity" site is measured (Ferguson-Miller et al., 1976). These observations differ greatly from those made previously by us in the absence of ATP, where there was as much as a 30-fold difference between the rates measured by the two methods (Smith et al., 1979b) with a similar preparation.

Discussion

ATP, ADP, and inorganic pyrophosphate, with or without Mg^{2+} ions, partially inhibit the oxidation of low concentrations of exogenous cytochrome *c* by different kinds of preparations of cytochrome oxidase, when assayed polarographically in buffers of nonbinding ions at pH 7.8. The inhibitory effect differs quantitatively among different kinds of preparations, and sometimes among different batches of one kind of preparation. The greatest effect on the reaction with exogenous cytochrome *c* was always seen with cytochrome *c* deficient SMP treated with DOC, where the oxidase remains membrane bound, but has maximal sites exposed for reaction with cytochrome *c* (Smith & Camerino, 1963). Usually the least inhibition was observed with purified cytochrome aa_3. This difference is not unexpected since we have shown (Davies et al., 1978) that different extents of reactive sites may be exposed in purified oxidase as compared with membrane-bound preparations. The Hartzell–Beinert preparation is reported to have a relatively low lipid content, but we have found that the Yonetani-type oxidase preparation reacts similarly (unpublished observations).

In contrast, the rate constants measured spectrophotometrically increased somewhat in the presence of ATP, ADP, or pyrophosphate. We feel that all of these effects can be explained by the binding of these compounds to cytochrome *c*, and the data extend our previous observations (Smith et al., 1979a,b) on the interaction of cytochrome *c* with cytochrome oxidase.

Our comparisons of the polarographic and spectrophotometric methods (Smith et al., 1979b) indicated that under some conditions the two methods measure different aspects of the oxidase reaction. In the spectrophotometric assays the cytochrome *c* must bind to and react with the oxidase and then dissociate in order for another molecule to react. In the polarographic assays, under conditions where the O_2 uptake rates are greatly in excess of the spectrophotometrically measured rates, the cytochrome *c* must remain bound for repeated reduction. We found that changing conditions affect the rates measured by the two methods in different ways, but these effects are not necessarily reciprocal, pointing to different kinds of combinations of cytochrome *c* with the oxidase under the different conditions. In agreement with this postulate, in the present experiments ATP and ADP had quantitatively similar effects on the spectrophotometric assays but different effects on the polarographic assays with SMP treated with DOC. Also, ATP inhibited the purified oxidase assayed polarographically but had little effect on the spectrophotometric assays in Tris–cacodylate buffer, pH 7.8.

Of the postulated different kinds of complexes formed between cytochrome *c* and cytochrome oxidase (Smith et al., 1979b), an especially reactive combination appears to be formed in buffers with nonbinding ions (Tris–cacodylate or Hepes) at pH 7.8, as shown by the high turnover rates in the polarographic assays as compared with the corresponding rates in the spectrophotometric assays. In the absence of ATP the O_2 uptake rates measured polarographically were as much as 30-fold greater than those calculated from the spectrophotometrically measured rates (Smith et al., 1979b, Figure 6). However, assay of a similar preparation in the presence of 3 mM ATP gave rates by the two methods that were not very different. This implies that binding of ATP or ADP to cytochrome *c* (Stellwagon & Shulman, 1973) inhibits the formation of the high turnover complex. The presence of higher concentrations of binding ions such as phosphate (Margalit & Schejter, 1973) has a similar effect (Smith et al., 1979b, and unpublished data). In agreement with this postulate is the observation that ATP had only a small inhibitory effect on O_2 uptake in the polarographic assays with exogenous cytochrome *c* at pH 6, where we found little or no extra O_2 uptake due to a high turnover complex (Smith et al., 1979b). In addition, ATP had less inhibitory effect on polarographic assays with purified cytochrome aa_3 than with SMP treated with DOC; we have found that the complex formed with the purified preparation in Tris–cacodylate, pH 7.8, has a lower turnover rate than that formed with the detergent-treated SMP (Davies et al., 1978).

ATP and ADP bind to horse cytochrome *c* in the immediate vicinity of histidine-26 (Stellwagon & Shulman, 1973), which is in the area around the heme crevice. Since various data indicate that the high-affinity binding site on cytochrome *c* for the oxidase is also in the same area (Smith et al., 1976; Smith et al., 1977; Ferguson-Miller et al., 1978), it is not surprising that binding of the nucleotides would inhibit the formation of a reactive complex.

Although the observations with ATP and ADP give additional insight into the interaction of cytochrome *c* and cytochrome oxidase, particularly in relation to the effect of binding ions on complex formation, the significance of these data for the regulation of O_2 uptake via cytochrome oxidase is not evident. The inhibitory effect on the reaction with low concentrations of added cytochrome *c* is only partial and is small in the presence of Mg^{2+} ions, in which combination the nucleotides exist predominantly within the cells. Also, the inhibitory effects of ATP and ADP are not very different and are additive rather than competitive. Any importance of these inhibitory effects would depend upon whether cytochrome *c* passes from its binding site on the reductase segment to the oxidase via solution, which is not known. In fact, there is some evidence that cytochrome *c* remains membrane bound during electron transport (Erecinska et al., 1975; Smith et al., 1979a). Even if it did dissociate into the intermembrane space, the partial inhibition observed would not be expected to inhibit overall electron transport of the mitochondrial system, since the oxidase activity is considerably greater than the reductase. Overall our data give no support to the idea that cellular concentrations of ATP and ADP regulate respiration by a direct effect on cytochrome oxidase activity.

The observation that ATP stimulates the NADH oxidase or the cytochrome oxidase activity with the endogenous cytochrome *c* of SMP (cytochrome *c* sufficient) is surprising. The endogenous cytochrome *c* must be retained by those SMP vesicles which are "inside out", so the site where cytochrome *c* reacts would not be exposed. However, our data disagree with the suggestion of Ferguson-Miller et al. (1976) that ATP has an *inhibitory* effect resulting from a structural change on binding of the nucleotide to the oxidase. Since the effect of ATP on the reaction of NADH oxidase and cytochrome oxidase with endogenous cytochrome *c* is similar, it looks like there may be some change in the membrane structure which increases the effectiveness of the reactions between the membrane-bound pigments. Also, the inhibition of the reaction

with exogenous cytochrome *c* is somewhat greater if the ATP is added after the reaction is initiated. Whatever the change resulting from its presence, it is not specific for ATP, since inorganic pyrophosphate shares these effects.

Acknowledgments

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References

Davies, H. C., Smith, L., & Nava, M. E. (1978) *Abstr. Int. Biophys. Congr., 6th*, 217.
Davies, H. C., Smith, L., & Nava, M. E. (1979) International Workshop on Membrane Bioenergetics, Detroit, July 1979.
Erecinska, M., Vanderkooi, J. M., & Wilson, D. (1975) *Arch. Biochem. Biophys. 171*, 108–116.
Ferguson-Miller, S., Brautigan, D. L., & Margoliash, E. (1976) *J. Biol. Chem. 251*, 1104–1115.
Hartzell, C. R., & Beinert, H. (1974) *Biochim. Biophys. Acta 368*, 318–338.
Lee, C. P., & Ernster, L. (1967) *Methods Enzymol. 10*, 543–548.
Margalit, P., & Schejter, A. (1973) *Eur. J. Biochem. 32*, 492–505.
Margoliash, E., & Frohwirt, N. (1959) *Biochem. J. 71*, 570–572.
Margoliash, E., & Walasek, O. (1967) *Methods Enzymol. 10*, 339–348.
Roberts, H., & Hess, B. (1977) *Biochim. Biophys. Acta 462*, 215–234.
Smith, H. T., Staudenmayer, N., & Millett, F. (1977) *Biochemistry 16*, 4971–4974.
Smith, L. (1978) *Methods Enzymol. 53 (Part D)*, 229–231.
Smith, L., & Conrad, H. (1956) *Arch. Biochem. Biophys. 63*, 403–413.
Smith, L., & Camerino, P. W. (1963) *Biochemistry 2*, 1432–1439.
Smith, L., Davies, H. C., & Nava, M. E. (1976) *Biochemistry 15*, 5827–5831.
Smith, L., Davies, H. C., & Nava, M. E. (1979a) in *Cytochrome Oxidase* (King, T. E., Orii, Y., Chance, B., & Okunuki, K., Eds.) pp 293–304, Elsevier/North Holland Press, Amsterdam.
Smith, L., Davies, H. C., & Nava, M. E. (1979b) *Biochemistry 18*, 3140–3146.
Stellwagon, E., & Shulman, R. G. (1973) *J. Mol. Biol. 75*, 683–695.
Vanneste, W. H. (1966) *Biochim. Biophys. Acta 113*, 175–178.

Cryoenzymology and Spectrophotometry of Pea Seedling Diamine Oxidase†

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ABSTRACT: Diamine oxidase follows bi-ter ping-pong kinetics, with an intermediate, "reduced" free-enzyme form being generated after the anaerobic conversion of amine to aldehyde. Visible spectra of diamine oxidase reacting at subzero temperatures provide evidence that this intermediate enzyme form is obtained via several other intermediates and that the environment of the Cu(II) changes dramatically during the course of the reaction [even though it is not reduced to Cu(I) during the catalytic cycle]. The spectrum of this form of diamine oxidase, which is obtained 0.5–2 h after the addition of amine at −5 to −15 °C, is independent of substrate, is identical with that obtained by anaerobic addition of substrate at room temperature, and provides evidence for a direct interaction of Cu(II) with the organic cofactor of the enzyme. This interaction is apparently charge transfer in nature. Upon removal of Cu(II) from the native enzyme, one obtains spectral evidence that the organic cofactor is still present. However, removal of the Cu(II) from the reduced (intermediate) enzyme form yields a featureless enzyme spectrum and a Cu(II)–chelate complex which contains a new ligand, which is presumably the second prosthetic group.

Diamine oxidase [DAO;[1] diamine:oxygen oxidoreductase (deaminating), EC 1.4.3.6] catalyzes the oxidative deamination of a variety of amines according to the general reaction

$$R\text{–}CH_2NH_2 + O_2 \rightarrow R\text{–}CHO + H_2O_2 + NH_3$$

where R equals $NH_2(CH_2)_3$– (putrescine), C_6H_5– (benzylamine), or 4-$(CH_3)_2NCH_2C_6H_4$– [*p*-[(dimethylamino)methyl]benzylamine]. The work described in this paper was performed by using pea seedling DAO; previous work has dealt with the porcine kidney enzyme (Kluetz & Schmidt, 1977a,b), which also attacks histamine but does not oxidize benzylamine.

DAO has been shown to follow bi-ter (uni uni uni bi) ping-pong kinetics [Bardsley et al., 1973; Nylēn & Szybek, 1974; notation and schematics of Cleland (1963)], in which 1 equiv of amine is converted to the corresponding aldehyde in an anaerobic stage, leaving the enzyme in an intermediate, "reduced" form (designated E′); the E′ form of DAO is subsequently reconverted to the native state upon reaction with O_2, eliminating H_2O_2 and NH_3:

amine	aldehyde	O_2	H_2O_2	NH_3
↓	↑	↓	↑	↑
E		E′		E

DAO requires as cofactors Cu(II) and an as yet unidentified organic moiety, and we have been particularly interested in

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[1] Abbreviations used: DAO, diamine oxidase; DAB, *p*-[(dimethylamino)methyl]benzylamine; PLP, pyridoxal 5′-phosphate; EDTA, ethylenediaminetetraacetic acid; Me_2SO, dimethyl sulfoxide; DDC, diethyldithiocarbamate.

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