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Reactions of Oxidizing and Reducing Radical Probes with Lipoamide Dehydrogenase†

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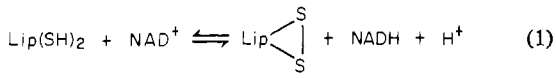
ABSTRACT: One-electron redox radicals generated by radiation–chemical methods have been reacted with the oxidized (E) form of pig heart lipoamide dehdyrogenase. The reducing radicals e_{aq}^- and $CO_2^-\cdot$ and $O_2^-\cdot$ do not measurably inactivate the enzyme, whereas the oxidizing species ·OH and $Br_2^-\cdot$ do. The $CO_2^-\cdot$ anion forms the semiquinone radical ·EH on the millisecond time scale, whereas at longer times only EH_2 is observed. Evidence suggests that $Br_2^-\cdot$ oxidizes adjacent sulfhydryl groups to form a disulfide in a manner similar to the reaction of Cu^{2+} ions. With ·OH, destruction of the flavin adenine dinucleotide (FAD) moiety is responsible for at least 50% of the enzyme inactivation. This destruction appears to be a result of secondary reactions which transfer damage from remote initial sites of attack to the flavin. Pathways for migration of e_{aq}^- damage also appear to exist.

Lipoamide dehydrogenase (EC 1.6.4.3) is the enzyme which catalyzes the oxidation of the dihydrolipoamide residue on lipoate acyltransferase to a lipoamide residue in the α-keto acid dehydrogenase multienzyme complexes (Reed, 1974). The overall reaction is shown in eq 1. The active site of the



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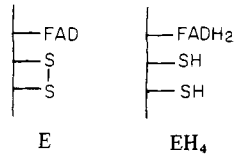
enzyme contains FAD[1] and an oxidation–reduction disulfide (Williams, 1976). Under mild conditions, the oxidized enzyme (E) can be reduced by two-electron equivalents to a species designated EH_2 which is the biological intermediate. However, under forcing conditions, EH_2 can be further reduced to an EH_4 form (Williams, 1976; Matthews & Williams, 1976). The spectral properties of these three species are quite different (Matthews & Williams, 1976). Those of E and EH_4 resemble fully oxidized and fully reduced FAD, respectively, since absorptions due to –SH and –S–S– groups are relatively weak in the 300–700-nm range of flavin absorption. These two forms can be written as



E EH_4

The structure of EH_2 cannot be represented so simply. Its spectrum has similarities to that of the semiquinone of FAD (although no electron paramagnetic resonance is observed) and to that of charge-transfer complexes involving FAD (Williams, 1976). The active-site disulfide is also thought to be reduced as AsO_2^- (Massey & Veeger, 1960; Searles & Sanadi, 1960), and trivalent organoarsenicals (McKay et al., 1979; Stevenson & Adamson, 1979) can bind there. Because of these conflicting data, EH_2 has been variously described as a biradical, a charge-transfer complex, and a covalently bound compound (Williams, 1976), viz.:

One possible approach to investigate electron-transfer pathways in the enzyme and to further elucidate the structure of EH_2 is to carry out one-electron oxidations or reductions on the various forms E, EH_2, and EH_4. In principle, this can be achieved by utilizing one-electron oxidizing and reducing species produced by pulse radiolysis, a technique which has sufficient time resolution to observe short-lived intermediates (Bielski & Gebicki, 1977; Adams & Wardman, 1977). Briefly, fast electrons set in motion by adsorbed γ rays or from an electron accelerator (Spinks & Woods, 1976) produce in dilute aqueous solutions ·OH, e_{aq}^-, and H· with yields of 2.8, 2.7, and 0.7 radicals per 100 eV, respectively (Adams & Wardman, 1977). The hydroxyl radical is the dominant species in N_2O-saturated solutions because of reaction 2. On

$$e_{aq}^- + N_2O + H_2O \rightarrow \cdot OH + OH^- + N_2 \qquad (2)$$

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[1] Abbreviations used: $LipS_2$, oxidized lipoamide; $Lip(SH)_2$, reduced lipoamide; FAD, flavin adenine dinucleotide; NAD, nicotinamide adenine dinucleotide; NADH, reduced NAD.

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addition of Br^- to these solutions, ·OH is converted to Br_2^-· which, like ·OH, is an oxidizing radical (eq 3). When formate

$$\cdot OH + 2Br^- \rightarrow Br_2^-\cdot + OH^- \quad (3)$$

is present in N_2O-saturated solution, all radicals are converted to the mildly reducing radical CO_2^-· via reactions 2 and 4.

$$H\cdot \text{ or } \cdot OH + HCO_2^- \rightarrow H_2 \text{ or } H_2O + CO_2^-\cdot \quad (4)$$

This in turn can be converted to O_2^-· in air-saturated solutions (eq 5). The above reactions are described in greater detail

$$e_{aq}^- \text{ or } CO_2^-\cdot + O_2 \rightarrow O_2^-\cdot \quad (5)$$

by Bielski & Gebicki (1977) and Adams & Wardman (1977).

In this paper we examine the specificity of the radicals Br_2^-·, CO_2^-·, O_2^-·, ·OH, and e_{aq}^- for reactions with the active site of the oxidized form of lipoamide dehydrogenase (E) and determine the extent of inactivation by these radicals. We also provide evidence for the production of a transient ·EH form of the enzyme.

Experimental Procedures

Materials. Pig heart lipoamide dehydrogenase, stabilized in ammonium sulfate solution, was obtained from a number of sources: Sigma Chemical Co., Calbiochem, and Boeringer. DL-Lipoamide was obtained from Sigma.

Methods. (1) Preparation of Lipoamide Dehydrogenase Solutions. Ammonium sulfate was removed from lipoamide dehydrogenase by dialysis against deionized water for 24 h at 4 °C. All experiments were performed at room temperature on solutions containing 4 mM sodium phosphate buffer and 4.5–6.5 μM lipoamide dehydrogenase [determined as per mol of enzyme-bound FAD, ϵ_{455} = 11 300 M^{-1} cm^{-1} (Matthews & Williams, 1976)]. When present, the sodium formate concentration was close to 20 mM, the potassium bromide concentration was 10 mM, and the lipoamide concentration was ~0.3 mM. All solutions were made up to volume with triply distilled water. The pH of these solutions was 6.8 ± 0.2.

The enzyme activity was measured by following the production of NADH spectrophotometrically at 360 nm from NAD^+ and dihydrolipoamide as outlined by Danson & Perham (1976). DL-Dihydrolipoamide was prepared from DL-lipoamide (Reed et al., 1958).

(2) Irradiations. γ irradiations were performed at room temperature in an AECL ^{60}Co "gammacell" with a steady dose rate of ~1850 rd min^{-1} (1.15 × 10^{17} eV mL^{-1} min^{-1}). When required, air was removed from the samples by bubbling with the appropriate gas, argon or nitrous oxide. The bubbling did not affect the enzyme activity. The spectra of the samples were recorded on a Pye-Unicam SP8-100 UV–visible spectrophotometer.

The yields for loss of enzyme activity are reported in terms of the radiation chemistry parameter *G* (Spinks & Woods, 1976; Armstrong & Buchanan, 1978). Thus *G*(inactivation) is the number of active sites destroyed per 100 eV of radiation energy absorbed by the solution. This is calculated from the loss of active sites per milliliter per minute as determined from enzyme assays and the dose rate in electronvolts per milliliter per minute.

Pulse radiolysis experiments were performed with a 4–5-μs pulse of electrons from a 1.5-MeV Van de Graaff generator as described earlier (Elliot et al., 1980). A 1 cm path length optical cell was used with a fresh solution for each pulse. Photolysis of the sample was minimized by only opening the light shutter immediately prior to the pulse.

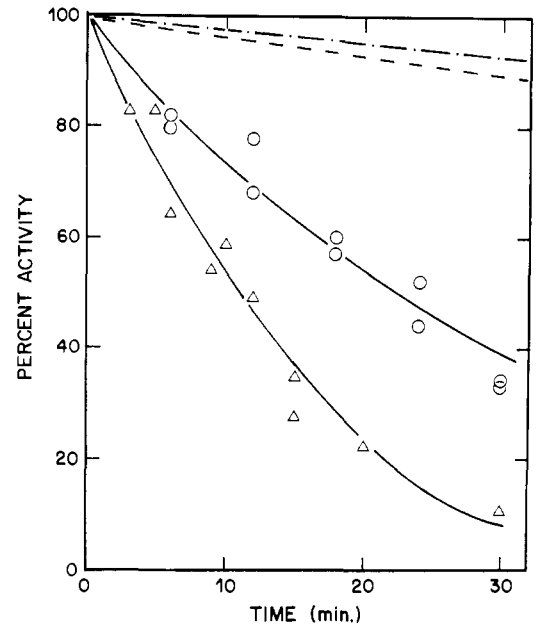


FIGURE 1: Inactivation plots of lipoamide dehydrogenase (5.5–6.5 μM) solutions: argon saturated, [e_{aq}^-, ·OH] (○); N_2O saturated, [·OH] (△); air saturated, 10 mM formate, [O_2^-·] (– – –); N_2O saturated, 10 mM formate, [CO_2^-·] (–·–). The last two solutions were irradiated up to 240 min.

Table I: Values for *G*(Inactivation) for Various Conditions of Radiolysis

conditions	radicals reacting and *G*(radical)[a]	*G*(inactivation)[b]
N_2O-saturated, 10 mM sodium formate	CO_2^-· (6.2)	0.008
air-saturated, 10 mM sodium formate	O_2^-· (6.2)	0.010
air-saturated	O_2^-· (3.5), ·OH (2.8)	0.066
argon-saturated	e_{aq}^- (2.7), ·OH (2.8), H· (0.7)	0.091
N_2O-saturated	·OH (5.5), H· (0.7)	0.15
N_2O-saturated, 10 mM KBr	Br_2^-· (5.5), H· (0.7)	0.19

[a] Number of radicals formed per 100 eV of radiation energy absorbed. [b] Number of active sites destroyed per 100 eV of radiation energy absorbed.

Results

Inactivation Studies. The percentage of lipoamide dehydrogenase activity remaining is plotted against time of exposure to radiation in Figure 1 for several radiation conditions. The values of *G*(inactivation) were calculated as described under Methods by using the rate of inactivation from the initial slopes of plots such as shown in Figure 1. They are summarized in Table I along with the yields per 100 eV of radical species for each set of conditions. From the results for solutions containing formate it is evident that CO_2^-· and O_2^-· are not very damaging to the enzyme. The results for the air-, argon-, and N_2O-saturated solutions indicate that e_{aq}^- and H· do not play a major role in the inactivation. The oxidizing radicals ·OH and Br_2^-·, however, are clearly the most lethal to the oxidized form (E) of the enzyme as can be seen in Table I.

Long-Term Spectral Changes. As a means of demonstrating whether the FAD/S–S moiety could be reduced reversibly by radiation–chemical methods, two different experiments were performed where oxidized lipoamide dehydrogenase was reacted with reducing species alone in air-free solutions con-

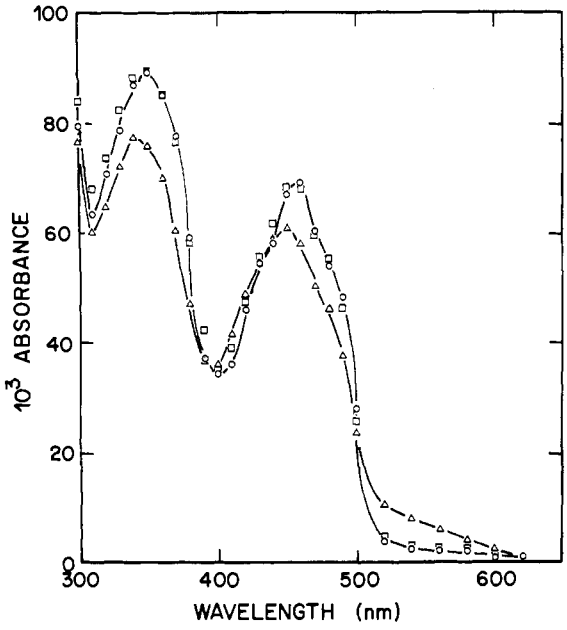
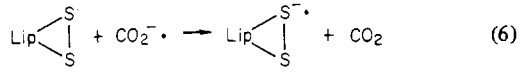


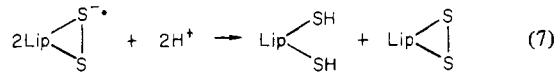
FIGURE 2: The spectra obtained from a N_2O-saturated solution containing 6.1 μM lipoamide dehydrogenase, 23 mM sodium formate, and 0.35 mM lipoamide before irradiation (O), after a 32-min γ irradiation (Δ), and after aeration of the irradiated solution ($\square$).

taining formate. In the first, lipoamide (0.35 mM) was added to scavenge the $CO_2^{-}\cdot$ radical (Faraggi et al., 1975; Chan et al., 1974; A. J. Elliot and D. A. Armstrong, unpublished data) and form the radical anion $LipS_2^{-}\cdot$ (eq 6). Since this anion



(6)

can transfer an electron to FAD (Chan et al., 1974), it may react directly with the enzyme. Alternately, it may disproportionate to form dihydrolipoamide (Faraggi et al., 1975; Chan et al., 1974) (eq 7). This is the substrate for the enzyme,



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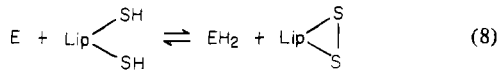
and reaction 8 would maintain the equilibrium concentrations of E, EH_2, and the oxidized and reduced forms of lipoamide (Matthews et al., 1977).



(8)

In Figure 2 the open circles show the initial spectrum of the lipoamide–E mixture. The large absorption near 350 nm is a combination of absorbance from the enzyme (see Figure 4) and lipoamide. The spectrum after the equivalent of 0.37 mM dihydrolipoamide had been produced is shown by the triangles. This concentration was calculated from the yield of $CO_2^{-}\cdot$ formed during 32 min of irradiation. The fall in the 455-nm peak, accompanied by the rise in the 520–600-nm range, is characteristic of the reduction of E to EH_2 (Matthews & Williams, 1976). The absorption near 350 nm also falls. This is, in part, due to the reduction of the lipoamide disulfide bond. Bubbling the irradiated solution with air restored the initial absorption (see Figure 2), showing that the enzyme and lipoamide were reversibly reduced and can be reoxidized.

In the second reduction experiment, the $CO_2^{-}\cdot$ radical anion was allowed to react directly with lipoamide dehydrogenase and the spectrum in the 300–650-nm range was taken after a 6-min irradiation which corresponds to the production of ~70 μM radicals. In Figure 3a, the difference spectrum

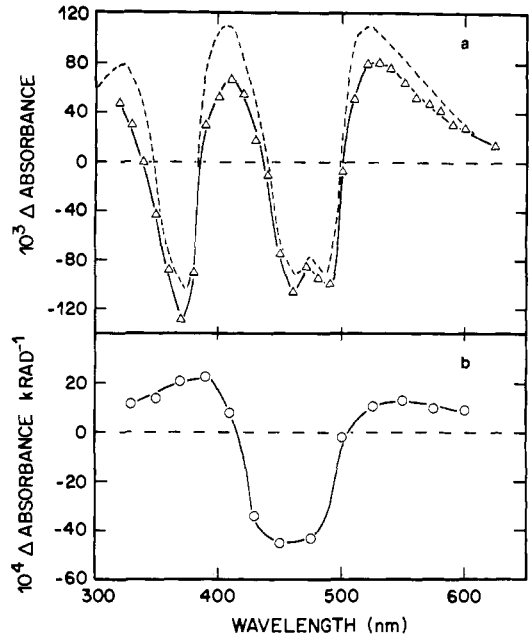


FIGURE 3: (a) Difference spectra between E and EH_2 after a 6-min γ irradiation of N_2O-saturated solution containing 5.8 μM lipoamide dehydrogenase and 17 mM sodium formate (Δ) and as calculated from Figure 4 of Matthews & Williams (1976) (---). (b) The difference spectrum obtained 700 μs after a N_2O-saturated solution containing 6.4 μM lipoamide dehydrogenase and 18 mM sodium formate was pulse irradiated (dose ~840 rd).

obtained in this experiment is compared to that calculated for E–EH_2 from Figure 4 of Matthews & Williams (1976). From a comparison of the amount of $CO_2^{-}\cdot$ formed to the magnitude of spectral changes at 520 nm [ϵ ~3000 M^{-1} cm^{-1}; Matthews & Williams (1976)] where only EH_2 absorbs, it is estimated that ~20% of $CO_2^{-}\cdot$ actually effect the reduction.

$$E + 2CO_2^{-}\cdot + 2H^+ \rightarrow EH_2 + 2CO_2 \qquad (9)$$

Bubbling the irradiated solution with air restored the original absorption, again confirming the reversibility of the reduction. These observations are similar to the previously observed reversible reduction of FAD by $CO_2^{-}\cdot$ (Land & Swallow, 1969).

When a N_2O-saturated solution of E was irradiated, the peaks belonging to the FAD moiety at 455 and 360 nm decreased while the absorbance below 340 nm increased (Figure 4a). These changes are due primarily to ·OH radicals (see Table I). Aeration of the irradiated solution caused the 360- and 455-nm peaks to increase slightly as well as a further increase in the absorbance below 340 nm. Irradiation of argon-saturated solutions gave results intermediate between solutions where $CO_2^{-}\cdot$ and ·OH were the reacting species. Some EH_2 was formed, presumably by reaction of e_{aq}^{-}, but this appeared to occur with very low efficiency. Bubbling with air caused EH_2 to revert to E as well as a further increase in the absorbance below 340 nm as observed in the N_2O case.

The spectrum obtained for a solution where $Br_2^{-}\cdot$ reacted with oxidized lipoamide dehydrogenase is shown in Figure 4b. The FAD portion of the spectrum changed on irradiation, with the shoulder at 480 nm diminished, whereas the intensity of the peak at 455 nm did not change. Furthermore, the absorbance at wavelengths below this peak increased, and none of these changes were reversed on bubbling with air.

Transient Spectral Changes. The transient spectra observed after pulse radiolysis of deoxygenated solutions containing the oxidized E form of lipoamide dehydrogenase are shown in Figure 3b and Figure 5. The difference spectrum for the reaction of $CO_2^{-}\cdot$ in Figure 3b was still increasing after 1 ms.

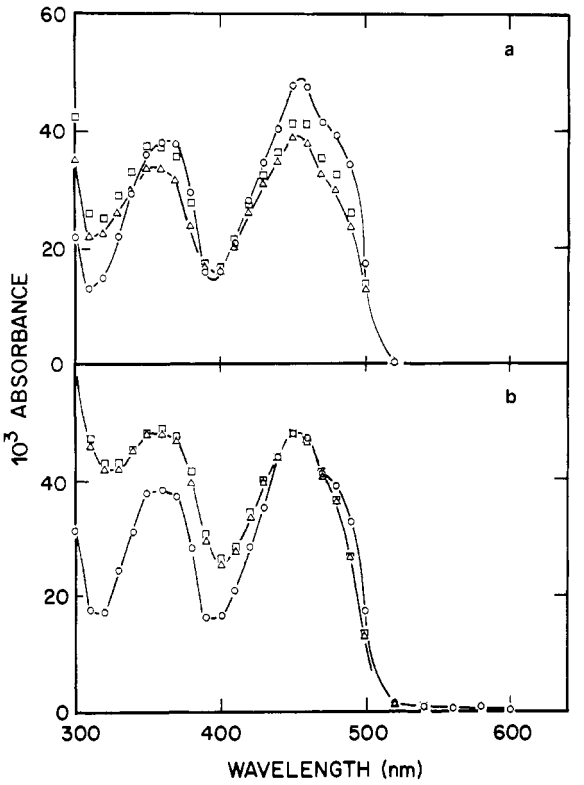


FIGURE 4: (a) Spectra obtained from a N_2O-saturated solution containing 4.1 μM lipoamide dehydrogenase before irradiation (○), after a 6-min irradiation (△), and after aeration of the irradiated solution (□). (b) The spectra obtained from a N_2O-saturated solution containing 4.1 μM lipoamide dehydrogenase and 20 mM KBr before irradiation (○), after 13 min of irradiation (△), and after aeration of the irradiated solution (□).

The dose used in this experiment was such that some $CO_2^{-}\cdot$ reacted by mutual disproportionation. Thus, an accurate value of the rate constant for the reaction of $CO_2^{-}\cdot$ could not be obtained. However, assuming a pseudo-first-order buildup of the signal, the rate constant was shown to be $<3 \times 10^8$ M^{-1} s^{-1}.

For N_2O-saturated solutions where ·OH was the principal attacking species, the spectrum was weak with increasing absorbance toward the ultraviolet region (triangles in Figure 5). In argon-saturated solutions where both e_{aq}^- and ·OH reacted with E, the intense absorption of e_{aq}^- was observed (Bielski & Gebicki, 1977) immediately following the pulse, and it decayed by reacting with the enzyme with a rate constant of $1.5 \pm 0.5 \times 10^{10}$ M^{-1} s^{-1}. After the decay of e_{aq}^- there was a net increase of absorbance below 425 and above 480 nm and a bleaching between 425 and 480 nm as shown by the hexagons in Figure 5. These changes, although weak, continued to increase well after e_{aq}^- decayed. The third and most intense spectrum in Figure 5 (circles) was formed by the reaction of $Br_2^{-}\cdot$ with E. The enzyme-derived transient decayed with mixed-order kinetics sufficiently fast (see insert in Figure 5) to make evaluation of the rate constant for reaction 10 difficult, but k_{10} was estimated from the pseudo-first-order decay of $Br_2^{-}\cdot$ and the known concentration of enzyme to be in the range $(2\text{–}6) \times 10^9$ M^{-1} s^{-1}.

$$Br_2^{-}\cdot + E \xrightarrow{k_{10}} (E\text{–transient}) \qquad (10)$$

Discussion

Oxidizing Radicals. The specificities of the three oxidizing radicals toward FAD and the various amino residues present in lipoamide dehydrogenase are quite different. $O_2^{-}\cdot$ is unreactive toward amino acids (Bielski & Shiue, 1979), although it appears to oxidize the SH group of cysteine. However, current evidence suggests that this is only important when the sulfhydryl is activated by its environment in the protein as is the case for papain or glyceraldehyde-3-phosphate dehydrogenase (Armstrong & Buchanan, 1978). Since the active-site sulfur atoms of E are already in the disulfide form, the small inactivation yield observed with $O_2^{-}\cdot$ (Table I) is in accord with expectations. In contrast to $O_2^{-}\cdot$, ·OH is reactive toward all amino acid residues (Farhataziz & Ross, 1977) and to FAD (A. J. Elliot and D. A. Armstrong, unpublished results). The $Br_2^{-}\cdot$ anion does not react at a measurable rate with FAD (Anderson et al., 1977; A. J. Elliot and D. A. Armstrong, unpublished results) and the aliphatic amino acids (Adams et al., 1972) but is quite reactive toward tryptophan, histidine, tyrosine, and the SH of cysteine (Adams et al., 1972).

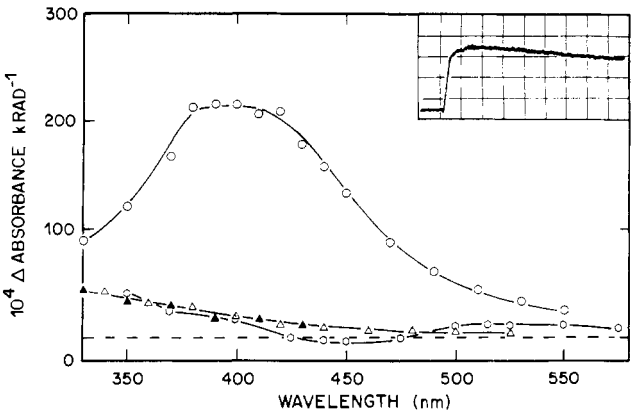
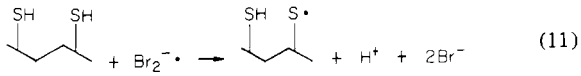


FIGURE 5: Spectra obtained when lipoamide dehydrogenase solutions (6.0–6.5 μM) were pulsed: N_2O saturated, [·OH], 50 μs after an ~800-rd (△) or 1334 rd (▲) pulse; argon flushed, [e_{aq}^-, ·OH], 80 μs after an ~650 rd pulse (○); N_2O saturated, 10 mM KBr, [$Br_2^{-}\cdot$], 156 μs after an ~760-rd pulse (○). (Insert) Time profile of the transient formed by reaction of $Br_2^{-}\cdot$ with lipoamide dehydrogenase at 440 nm, time = 20 μs/division, 1 vertical division = 4.7×10^{-3} absorbancy unit, and dose = 740 rd.

The pulse radiolysis experiment indicates that $Br_2^{-}\cdot$ forms a transient with λ_{max} near 400 nm (circles in Figure 5). Assuming 100% efficiency for its formation (i.e., 5.6×10^{-6} M per krd), the extinction coefficient taken from the absorption at 156 μs was 3900 M^{-1} cm^{-1}. This represents a minimum value since the transient was decaying, as can be seen in the insert in Figure 5. Of the residues present in lipoamide dehydrogenase (Williams, 1976), only histidine and tyrosine form transients with absorptions centered near 400 nm (Adams et al., 1972), and both have extinction coefficients $\ll$3900 M^{-1} cm^{-1} [ϵ = 1200–2000 M^{-1} cm^{-1} for histidine (Bansal & Sellers, 1975; Faraggi & Bettelheim, 1977; Feitelson & Hayon, 1973) and ϵ = 2700–3200 M^{-1} cm^{-1} for tyrosine (Feitelson & Hayon, 1973; Bansal & Fessenden, 1976)]. Furthermore, $Br_2^{-}\cdot$ does not react with free FAD and it is necessary to look for alternative explanations for the strong 400-nm transient.

The E form of pig heart lipoamide dehydrogenase has seven to eight free SH groups, two of which are close enough to form a S–S bond. The oxidation of these two SH groups to form a disulfide can be effected, for example, by Cu^{2+} (Matthews & Williams, 1974). This feature, the known reactivity of $Br_2^{-}\cdot$ toward SH (Adams et al., 1972), and the well-documented ionization equilibrium of radicals of disulfhydryl molecules (Bielski & Gebicki, 1977; Chan et al., 1974) suggest that the following reaction sequence occurred:



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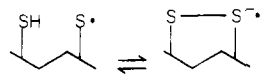

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Disulfide anions have strong absorptions [ϵ = 5000–15 000 M^{-1} cm^{-1} peaking in the range 390–450 nm (Faraggi et al., 1975; Chan et al., 1974; Hoffman & Hayon, 1972; Redpath, 1973; Purdie et al., 1973)]. Those tending toward a cis orientation, such as oxidized lipoic acid and oxidized dithiothreitol, absorb near 400 nm as in the present instance. Oxidation of neighboring sulfhydryl groups by Cu^{2+} to form a disulfide is known to inactivate lipoamide dehydrogenase (Casola et al., 1966). The relatively high G(inactivation) observed with $Br_2^{-}\cdot$ would therefore be consistent with the production of S–$S^{-}\cdot$ (reactions 11 and 12) followed by electron transfer to some other residue. However, this residue could not be FAD since in Figure 4b there is no growth in the region above 500 nm or decrease in the 455-nm peak which would accompany the reduction of FAD. The disappearance of the shoulder at 480 nm (Figure 4b) implies that $Br_2^{-}\cdot$ caused changes to the enzyme structure that placed FAD in a more hydrophilic environment (Palmer & Massey, 1968), possibly as a result of disulfide formation. The strong increase in absorption below 450 nm indicates that other changes in the enzyme also occurred.

The enzyme is also inactivated by reaction with ·OH (Figure 1 and Table I). In this case one can see that irreversible damage has occurred to the FAD moiety since the 455-nm peak is decreased, and aeration of the γ-irradiated solution does not restore the FAD absorption completely (Figure 4a). For a 6-min irradiation, the destruction of FAD, as estimated from the absorption after aeration and assuming no absorbance from destroyed FAD at 455 nm, would account for ~50% of the inactivation. This percentage would be higher if the product of the FAD reaction also absorbed at 455 nm.

The direct reaction of ·OH with FAD produces a strong bleaching of the 455-nm peak on the microsecond time scale (A. J. Elliot and D. A. Armstrong, unpublished results), but, as can be seen from the triangles in Figure 5, this did not occur following ·OH reaction with E. This indicates that direct reaction between ·OH and the enzyme-bound FAD was not significant and that destruction of the FAD on the long (i.e., γ radiolysis) time scale arose from secondary reactions of radicals formed elsewhere.

The relatively featureless spectrum for the OH-produced transients above 330 nm is largely due to the lack of specificity of this radical. While the observed absorption is predominantly due to OH reactions with tryptophyl, tyrosyl, and histidyl residues (Armstrong & Swallow, 1969; Feitelson & Hayon, 1973; Bansal & Sellars, 1975; Rao et al., 1975; Faraggi & Bettelheim, 1977), one must realize that reactions at other residues, which produce negligible absorption in this region, will also occur. Of potential importance is reaction with the free sulfhydryl located at the NAD^+ binding site (Matthews & Williams, 1974). Alkylation of this group by iodoacetamide destroys the enzyme activity. Its modification by ·OH (or $Br_2^{-}\cdot$) would do the same yet the absorbance of the cysteine RS· transient would be undetected [λ_{max} ~330 nm, ϵ_{max} ~400 M^{-1} cm^{-1}; Hoffman & Hayon (1973)].

Reducing Radicals. In the pulse radiolysis experiments, the species formed initially by $CO_2^{-}\cdot$ was not EH_2 as can be seen by comparing the difference spectra in Figure 3. In the active site there are two groups which can be reduced by $CO_2^{-}\cdot$, namely, the disulfide (Chan et al., 1974; Faraggi et al., 1975) and FAD (Land & Swallow, 1969; Anderson, 1976). As disulfide groups do not absorb appreciably above 330 nm and since S–$S^{-}\cdot$ absorbs strongly with a λ_{max} = 390–450 nm (Faraggi et al., 1975; Chan et al., 1974; Redpath, 1973; Purdie et al., 1973; Hoffman & Hayon, 1972), a transient absorption with the λ_{max} = 390–450 nm would have been expected if $CO_2^{-}\cdot$ reacted with the disulfide. This was not observed. However, bleaching of the 450-nm FAD absorption did occur as can be seen in Figure 3b.

This difference spectrum in Figure 3b is similar to those between flavoproteins and their neutral semiquinone forms (Faraggi & Klapper, 1979; Massey & Ghisla, 1974; Massey & Palmer, 1966), indicating that it is the semiquinone form ·EH that has been produced. This is the first time the semiquinone form of lipoamide dehydrogenase has been reported because on the time scale of the earlier experiments it was not stable (Massey & Palmer, 1966).

The pulse radiolysis experiments in argon-saturated solutions, where ·OH and e_{aq}^{-} are the principal reacting species (hexagons in Figure 5), also showed a bleaching between 425 and 480 nm and a growth above 480 nm. Since these changes cannot be ascribed to ·OH (see triangles in Figure 5), it appears the e_{aq}^{-} can also produce ·EH. However, from the intensity of the absorptions the yield of ·EH is smaller than with $CO_2^{-}\cdot$.

The continued growth of ·EH absorption after the disappearance of e_{aq}^{-} is another manifestation of the migration of a damage site from one point to another on the enzyme. However, it occurred on a microsecond time scale, whereas the migration of the ·OH damage to the flavin noted above occurs much more slowly.

The fact that only EH_2 was observed after the long time scale γ radiolysis with $CO_2^{-}\cdot$ implies either that ·EH disproportionates to E and EH_2 or that it reacts with a second $CO_2^{-}\cdot$.

$$\cdot EH + CO_2^{-}\cdot + H^+ \rightarrow EH_2 + CO_2 \qquad (13)$$

The low efficiency for the formation of EH_2 in the γ-radiolysis experiments demonstrates that some $CO_2^{-}\cdot$ may react at sites other than FAD or the active site disulfide. However, such processes have a negligible effect on the activity of the enzyme since the value of G(inactivation) with $CO_2^{-}\cdot$ is extremely small. Similar conclusions apply to the reactions of $e_{aq}^{-}\cdot$ and, in contrast to its sensitivity to ·OH and $Br_2^{-}\cdot$, the E form of lipoamide dehydrogenase is not inactivated by either of the reducing radicals significantly.

References

Adams, G. E., & Wardman, P. (1977) in *Free Radicals in Biology* (Pryor, W. A., Ed.) Vol. 3, pp 53–95, Academic Press, New York.

Adams, G. E., Aldrich, J. E., Bisby, P. H., Cundall, R. B., Redpath, J. L., & Willson, R. L. (1972) *Radiat. Res. 47*, 278.

Anderson, R. F. (1976) *Chem. Ber. 80*, 969.

Anderson, R. F., Patel, K. B., & Adams, G. E. (1977) *Int. J. Radiat. Biol. Relat. Stud. Phys., Chem. Med. 32*, 523.

Armstrong, D. A., & Buchanan, J. D. (1978) *Photochem. Photobiol. 28*, 743.

Armstrong, R. C., & Swallow, A. J. (1969) *Radiat. Res. 40*, 563.

Bansal, K. M., & Sellers, R. M. (1975) *J. Phys. Chem. 79*, 1775.

Bansal, K. M., & Fessenden, R. W. (1976) *Radiat. Res. 67*, 1.

Bielski, B. H. J., & Gebicki, J. M. (1977) in *Free Radicals in Biology* (Pryor, W. A., Ed.) Vol. 3, p 1, Academic Press, New York.

Bielski, B. H. J., & Shiue, C. C. (1979) in *Oxygen Free Radicals and Tissue Damage*, pp 43–48, Excerpta Medica.

Casola, L., Brumby, P. E., & Massey, V. (1966) *J. Biol. Chem. 241*, 4977.
Chan, S. W., Chan, P. C., & Bielski, B. H. J. (1974) *Biochim. Biophys. Acta 388*, 213.
Danson, M. J., & Perham, R. N. (1976) *Biochem. J. 159*, 677.
Elliot, A. J., Wilkinson, F., & Armstrong, D. A. (1980) *Int. J. Radiat. Biol. Relat. Stud. Phys., Chem. Med. 38*, 1.
Faraggi, M., & Bettelheim, A. (1977) *Radiat. Res. 71*, 311.
Faraggi, M., & Klapper, M. H. (1979) *J. Biol. Chem. 254*, 8139.
Faraggi, M., Redpath, J. L., & Tal, Y. (1975) *Radiat. Res. 64*, 452.
Farhataziz & Ross, A. B. (1977) *Natl. Stand. Ref. Data Ser. (U.S. Natl. Bur. Stand.) 59*.
Feitelson, J., & Hayon, E. (1973) *J. Phys. Chem. 77*, 10.
Hoffman, M. Z., & Hayon, E. (1972) *J. Am. Chem. Soc. 94*, 7950.
Hoffman, M. Z., & Hayon, E. (1973) *J. Phys. Chem. 77*, 990.
Land, E. J., & Swallow, A. J. (1969) *Biochemistry 8*, 2117.
Matthews, R. G., & Williams, C. H., Jr. (1974) *Biochim. Biophys. Acta 370*, 39.
Matthews, R. G., & Williams, C. H., Jr. (1976) *J. Biol. Chem. 251*, 3956.
Matthews, R. G., Ballou, D. P., Thorpe, C., & Williams, C. H., Jr. (1977) *J. Biol. Chem. 252*, 3199.
Massey, V., & Veeger, C. (1960) *Biochim. Biophys. Acta 40*, 184.
Massey, V., & Palmer, G. (1966) *Biochemistry 5*, 3181.
Massey, V., & Ghisla, S. (1974) *Ann. N.Y. Acad. Sci. 227*, 446.
McKay, D. J., Robinson, J. A., & Stevenson, K. J. (1979) *Proc. Int. Congr. Biochem., 11th 11*, 186.
Palmer, G., & Massey, V. (1968) in *Biological Oxidations* (Singer, T. P., Ed.) pp 263–300, Wiley-Interscience, New York.
Purdie, J. W., Gillis, H. A., & Klassen, N. V. (1973) *Can. J. Chem. 51*, 3132.
Rao, P. S., Simic, M., & Hayon, E. (1975) *J. Phys. Chem. 79*, 1260.
Redpath, J. L. (1973) *Radiat. Res. 51*, 364.
Reed, L. J. (1974) *Acc. Chem. Res. 7*, 40.
Reed, L. J., Koike, M., Levitch, M. E., & Leach, F. R. (1958) *J. Biol. Chem. 232*, 143.
Searles, R. L., & Sanadi, D. R. (1960) *Biochem. Biophys. Res. Commun. 2*, 189.
Spinks, J. W. T., & Woods, R. J. (1976) in *An Introduction to Radiation Chemistry*, Wiley, New York.
Stevenson, K. J., & Adamson, S. R. (1979) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 38*, 454.
Williams, C. H., Jr. (1976) *Enzymes, 3rd Ed. 13*, 89.

Study of 3α,20β-Hydroxysteroid Dehydrogenase with an Enzyme-Generated Affinity Alkylator: Dual Enzyme Activity at a Single Active Site†

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ABSTRACT: The substrate 17β-[(1*S*)-1-hydroxy-2-propynyl]-androst-4-en-3-one (β-HPA) and its enzyme-generated alkylating product 17β-(1-oxo-2-propynyl)androst-4-en-3-one (OPA) were synthesized to investigate the relationship between the 3α and 20β activities observed in commercially available cortisone reductase (EC 1.1.1.53) from *Streptomyces hydrogenans*. β-HPA, a substrate [apparent K_m = 145 μM; V_{max} = 63 nmol (min μg)$^{-1}$], when enzymatically oxidized by cortisone reductase to OPA, inactivates simultaneously the 3α and 20β activities in a time-dependent and irreversible manner following pseudo-first-order kinetics. OPA alone, an affinity alkylating steroid (K_I = 40.5 μM; k_3 = 1.8 × 10^{-2} s^{-1}), simultaneously inactivates 3α and 20β activities in a time-dependent and irreversible manner. At pH 7, the $t_{1/2}$ of enzyme inactivation for β-HPA (10 h) or OPA (41 min) is slower than at pH 9.2 (β-HPA, 16 min, and OPA, 3.3 min). Substrates (progesterone, 20β-hydroxypregn-4-en-3-one, and 5α-dihydrotestosterone), but not all steroids (20α-Δ^4-pregn-4-en-3-one and 17β-estradiol), protect against loss of both enzyme activities by β-HPA and OPA. The α isomer of HPA is not enzymatically oxidized and therefore does not cause inactivation of either 3α or 20β activity. Thus, β-HPA functions as a substrate for the enzymatic generation of a powerful affinity alkylator of cortisone reductase. Second, the identical change in both the 3α and 20β activities in all experimental conditions clearly results from dual enzyme activity at a single enzyme active site.

The oxidoreductase 20β-hydroxysteroid dehydrogenase (20β-HSD) (EC 1.1.1.53; trivial name, cortisone reductase) is officially named as specific for 20-ketopregnene steroids. Pocklington & Jeffrey (1968) observed reaction with 3-keto-androstane steroids and initiated several reports of 3-oxido-reductase activity (Gibb & Jeffery, 1971, 1972, 1973). These kinetic studies did not resolve whether catalysis occurred at one or at different active centers. Blomquist (1973) demonstrated comigration of the 3α and 20β activities in disc gel electrophoresis, thereby excluding a second enzyme as an explanation for two activities. Edwards & Orr (1978) reported that inactivation studies using haloacetoxy steroid derivatives showed interacting, perhaps even overlapping, 3- and 20-ketosteroid binding sites.

This report describes the inactivation of cortisone reductase, with the simultaneous loss of the 3α- and 20β-oxidoreductase activities when the substrate, 17β-[(1*S*)-1-hydroxy-2-

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