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Yeast and Horse Liver Alcohol Dehydrogenases: Potential Problems in Target Size Analysis and Evidence for a Monomer Active Unit†

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ABSTRACT: Yeast and horse alcohol dehydrogenases are commonly used as standards for radiation inactivation analysis of proteins, usually assuming that the minimal functional unit corresponds to the physical size in solution, a tetramer (M_r = 148 000) and a dimer (M_r = 80 000), respectively. Results described in this paper demonstrate that molecular weight overestimates may be obtained for the yeast protein as a result of its unusual sensitivity to secondary radiation products. Irradiation in the presence of sulfhydryl reagents results in a smaller functional size estimate (67 000 ± 3000) than that obtained in their absence (128 000 ± 5000), indicating that some sulfhydryl groups in the enzyme may be particularly susceptible to attack by radiolytic species. Analysis of the horse liver enzyme reveals that although it has structural and functional similarities to the yeast protein, it is not as prone to secondary radiation damage and gives a minimal functional size estimate (33 000 ± 1000) that most closely corresponds to a monomer. Quantitation of disappearance of the protein from a sodium dodecyl sulfate gel as a function of radiation dose also gives a target size (48 000 ± 3000) in reasonable agreement with the monomer molecular weight. These results indicate that the individual subunits of horse liver alcohol dehydrogenase have independent catalytic capacity and imply that the same may be true for the yeast enzyme.

Radiation inactivation or target size analysis is a technique that has been widely used to determine the functional molecular weight of proteins in pure and impure states. The functional size determined by this method often corresponds well with the actual physical molecular weight measured by amino acid sequence analysis or by hydrodynamic methods (Kempner & Schlegel, 1979; Shikita & Hatano-Sato, 1972; Lowe & Kempner, 1982; Kempner & Haigler, 1982; Haigler et al., 1985; Suarez, 1986; Kempner, 1987). In the case of multisubunit enzymes, information may be obtained regarding the catalytic capacity of individual subunits within an ag-

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gregate form. Such information is often difficult to obtain by other biochemical or biophysical methods.

In spite of considerable evidence indicating the validity of the application of target theory to the analysis of radiation inactivation of proteins, a number of theoretical and practical problems remain. One of these relates to the generation of secondary radiation products in the solvent, which may cause a loss of activity in addition to that produced by the direct action of the high-energy ionizing particles. Although this does not appear to be a major problem for most enzymes at the low temperatures at which irradiation is usually carried out, a striking example is provided by yeast alcohol dehydrogenase, an enzyme commonly used as a standard in target analysis (Lo et al., 1982; Lilly et al., 1983; Venter, 1983; Venter et al., 1983).

Yeast alcohol dehydrogenase exists as a tetramer of molecular weight 148 000 in the purified soluble form (Buhner & Sund, 1969), and it has often been assumed that the aggregate of four identical subunits (each 37 000; Wills & Jornvall, 1979) is the functional unit (Lo et al., 1982). Some evidence from target analysis supports this idea (Venter et al., 1983), but other results indicate that a monomer (Hutchinson et al., 1957) or a dimer (Shikita & Hatano-Sato, 1972; McIntyre & Churchill, 1985) is the minimal active unit. Our studies suggest that these inconsistencies are due to an unusual sensitivity of the enzyme to damage by secondary radiation products, since the activity of the protein is dependent on several highly reactive sulfhydryl groups that are involved in binding readily dissociable zinc atoms (Branden et al., 1975; Twu et al., 1973; Leskovac & Parkov-Pericin, 1975; Belke et al., 1974; Klinman & Welsh, 1976). This unusual sensitivity is revealed by anomalous radiation inactivation plots and can be minimized by addition of sulfhydryl reagents during irradiation. Nevertheless, the functional size estimated for the yeast enzyme remains questionable because the degree of protection achieved cannot be assessed. In an attempt to clarify the results obtained with yeast alcohol dehydrogenase, we also studied the radiation inactiviation behavior of the horse liver enzyme. This closely related protein is isolated as a dimer (Branden et al., 1975), has somewhat less reactive sulfhydryls (Reynolds & McKinley-McKee, 1975), and has less tendency to lose the two zinc atoms bound per 40 000 molecular weight subunit (Klinman et al., 1977).

The results of these studies indicate that the minimal functional unit of horse liver alcohol dehydrogenase is a monomer, implying that the same may be true of the yeast enzyme.

Experimental Procedures

Reagents. Butylated hydroxytoluene (BHT; Sigma), desferal (deferoxamine mesylate; CIBA Pharmaceutical Co.), horse liver alcohol dehydrogenase (Sigma), bakers' yeast alcohol dehydrogenase (Sigma A-3263, lot 81F-8210), ethanol (absolute; Asper Alcohol and Chemical Co.), nicotinamide adenine dinucleotide (NAD; Sigma), thioglycerol (Sigma), dithiothreitol (Sigma), sodium dodecyl sulfate (SDS; Pierce), Brilliant Blue (Sigma), and bovine serum albumin (BSA; Sigma, fraction V) were obtained from the indicated sources.

Irradiation Conditions. Radiation was performed at the Armed Forces Radiobiology Research Institute, Bethesda, MD, with the experimental setup described by Harmon et al. (1985). The samples were prepared by placing a 200-μL aliquot in a glass ampule, freezing in liquid nitrogen, purging with nitrogen gas, and rapidly sealing with an oxygen torch. Yeast alcohol dehydrogenase (a lyophilized powder containing <2% citrate salts) was dissolved in water at 1 mg/mL and irradiated in the presence or absence of 1 mg/mL BSA. Horse liver alcohol dehydrogenase (a lyophilized powder containing <2% phosphate salts) was diluted in water to 7 mg/mL and frozen in the presence of 1 mg/mL BSA for irradiation. Radiation dose was measured with thermoluminescent dosimeters to determine beam intensity before and after sample irradiation (Harmon et al., 1985).

Enzyme Assays. Yeast alcohol dehydrogenase was assayed spectrophotometrically according to Vallee and Hoch (1955) at 0.39 M ethanol. Details of dilution and assay buffer conditions are given in the legends to Figures 1 and 3. Horse liver alcohol dehydrogenase was assayed by the same procedure but at a lower ethanol concentration (7.7 mM). Other assay conditions are specified in the legend to Figure 4.

SDS–Polyacrylamide Gel Electrophoresis. The amount of horse liver alcohol dehydrogenase and bovine serum albumin remaining in irradiated samples was examined by electrophoresis in 10% acrylamide gels (with a 5% acrylamide stacking gel) using the discontinuous buffer system of Laemmli (1970), followed by Brilliant Blue staining. Samples were prepared by diluting the irradiated proteins 1:14 into a buffer with a final composition of 47 mM tris(hydroxymethyl)-aminomethane hydrochloride (Tris-HCl), 7.5% glycerol, 2.25% sodium dodecyl sulfate, 3.75% β-mercaptoethanol, and 0.04% Bromophenol Blue, pH 6.8. The samples were boiled for 3 min, and 10 μL of each was applied to the gel. Electrophoresis was carried out by using a Bio-Rad vertical slab gel apparatus with cooling. After electrophoresis, the gels were fixed for 1 h in 10% acetic acid and then stained for 2 h in 0.25% Brilliant Blue, 50% methanol, and 7.5% acetic acid. Destaining was performed overnight in 25% methanol and 10% acetic acid.

Samples of yeast and horse liver alcohol dehydrogenases were also run on an SDS–polyacrylamide gel system as described by Kadenbach et al. (1983). Gels were scanned at 575 nm by using a Gelman Model ACD-18 densitometer with automatic integrating capacity.

Treatment of Data and Calculation of Molecular Weights. Each point on the radiation inactivation curves represents the V_{max} of a single irradiated sample, obtained from multiple assays per vial at 5–10 different substrate concentrations. The type of data used is illustrated in Figure 2. The V_{max} values were determined by using a computer program based on the Wilkinson method of analysis (Wilkinson, 1961) as described by Brooks and Suelter (1986). Where error bars are included, they represent the standard deviation of the V_{max} determined by this program from the variance of the line fitted to the kinetic data.

Molecular weights were calculated from the equation

$$M = (6.4 \times 10^{11})S_t/D_{37}$$

where M is the molecular mass of the protein, D_{37} is the dose of radiation in rads required to reduce the sample activity (or the protein concentration) to 37% of its original value, and S_t is the correction factor for the temperature of irradiation (Kempner & Haigler, 1982; at −135 °C = 2.8).

Results

Anomalous Inactivation Behavior of Yeast Alcohol Dehydrogenase. Yeast alcohol dehydrogenase was irradiated in sealed vials at −135 °C as described under Experimental Procedures. After irradiation, samples were transported on dry ice and stored in liquid nitrogen. Immediately prior to assay, individual samples were thawed and diluted sufficiently to allow kinetic analysis over a range of NAD concentrations. The K_m values for NAD did not change significantly as a function of dose. The V_{max} values obtained from these analyses

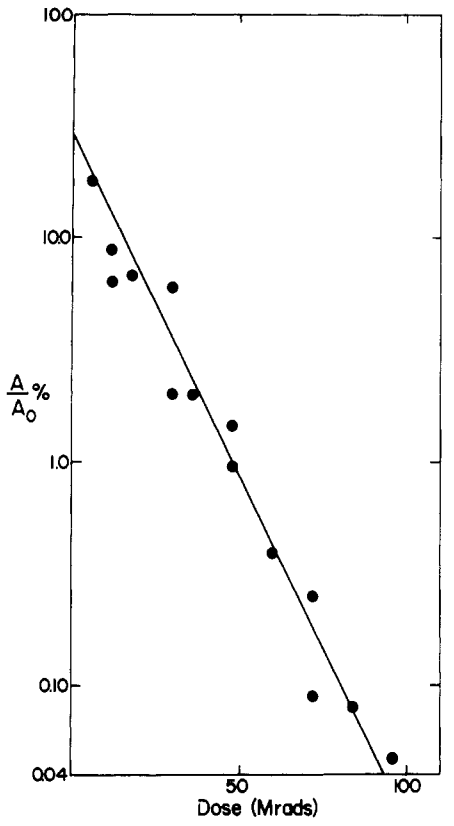

FIGURE 1: Plot of surviving activity of yeast alcohol dehydrogenase after irradiation in sealed vials with 13-MeV electrons at −135 °C. Each point represents the maximal velocity obtained from multiple (5–10) assays of a single sample, analyzed as described under Experimental Procedures. The irradiated samples contained 1 mg/mL yeast alcohol dehydrogenase (Sigma; 200 units mg^{-1} mL^{-1}). Activity was measured spectrophotometrically as described under Experimental Procedures, after dilution into 0.01 M phosphate buffer at pH 7.5 containing 0.1% gelatin. The molecular weight (128 000) was calculated as described under Experimental Procedures. If a least-squares analysis of the data was constrained to pass through 100% of the original activity, an estimate of 150 000 was obtained.

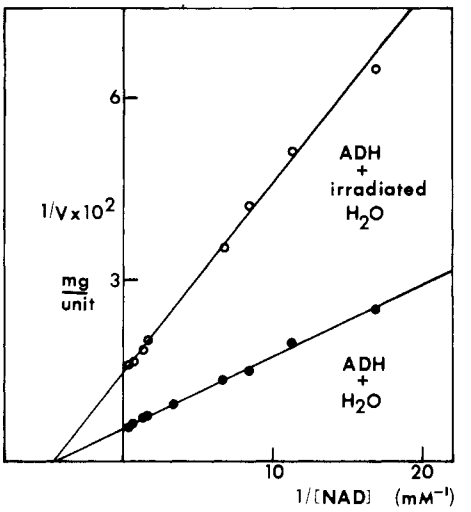

FIGURE 2: Comparison of Lineweaver–Burk plots of kinetics of the reduction of NAD by yeast alcohol dehydrogenase after dilution into irradiated (○) and unirradiated water (●). Water was exposed to 3 Mrad and kept in liquid nitrogen prior to addition to the enzyme. Activity was measured as described under Experimental Procedures.

were used to calculate the fraction of surviving activity at each radiation dose. As shown in Figure 1, the decay in yeast alcohol dehydrogenase activity after irradiation at −135 °C could be described by a simple exponential, but the resulting curve extrapolated to 30% instead of 100% of the initial activity. When the data were normalized to this lower apparent initial activity, a molecular weight of 128 000 was obtained. However, the large drop in activity with very low doses suggested that either a very large molecular weight species was responsible for 70% of the activity or significant damage was occurring as a result of the action of radicals produced secondarily to ionization events in the solvent. To test this latter possibility, samples of water were exposed to 3- and 48-Mrad doses of irradiation in sealed vials at −135 °C under the same conditions used for the enzyme. Unirradiated enzyme was then rapidly mixed with the irradiated water as it thawed, and the kinetic behavior was compared to a sample of enzyme diluted in a similar way into unirradiated water. Exposure of the enzyme to water irradiated with 3 or 48 Mrad resulted in a 70% decrease in maximal velocity with no detectable change in K_m for NAD (Figure 2). These results demonstrate that secondary radiation products accumulated even at low temperature, that they can cause dramatic loss of activity in the case of this enzyme after thawing, and that they could account for the zero-dose intercept at 30% rather than 100% activity.

Protective Effects of Thiol Reagents. The possibility was considered that the unusual sensitivity of yeast alcohol dehydrogenase to secondary radiation effects could be due to the presence of a large number of sulfhydryl groups in the enzyme (eight per subunit), several of which are essential for activity (Twu et al., 1973; Leskovac & Pavkov-Pericin, 1975) and highly susceptible to attack by radiolytic products (Armstrong & Buchanan, 1978). Therefore, the enzyme was irradiated in the presence of excess thioglycerol to determine if protection of sensitive sulfhydryls could be achieved. Initial experiments showed that the presence of 10 mM thioglycerol during irradiation did result in higher levels of activity immediately after thawing, but a time-dependent loss of activity was observed during kinetic analysis, concomitant with evolution of bubbles with a strong smell of sulfur from the samples. To stabilize the enzyme, it was necessary to add thioglycerol not only to the sample during irradiation but also to the thawing and diluted samples, especially at the higher doses of irradiation. As shown in Figure 3A (upper line), the presence of thioglycerol during irradiation, and additional thioglycerol during thawing, prevented the initial loss of activity and changed the slope of the inactivation curve in the direction of a smaller molecular weight (M_r = 67 000 ± 3000). It should be noted that the conditions of this experiment differ from those used to obtain the data shown in Figure 1, in that BSA was present in all solutions. This may account for the higher extrapolated zero-dose activity (80% of the original activity) found for the control enzyme in the absence of thioglycerol (Figure 3A, lower line). Nevertheless, the calculated molecular weight (117 000) was very similar to that obtained from the data in Figure 1.

Since very high levels of thioglycerol appeared to be necessary to stabilize the enzyme after thawing, a more effective protective agent was sought. Some evidence suggested that dithiothreitol might have superior protective properties (Armstrong & Buchanan, 1978). However, as shown in Figure 3B (upper line), the levels of activity observed after irradiation in the presence of dithiothreitol were lower than those observed in the presence of thioglycerol, and the former reagent was also not as effective as thioglycerol in preventing time-dependent loss of activity after thawing. If high concentrations of thioglycerol or dithiothreitol were present only in the dilution and assay buffers but not during irradiation, no protection or repair was observed (Figure 3B, lower line). At the levels used (see legend to Figure 3B), these reagents did not inhibit the activity of the unirradiated alcohol dehydrogenase.

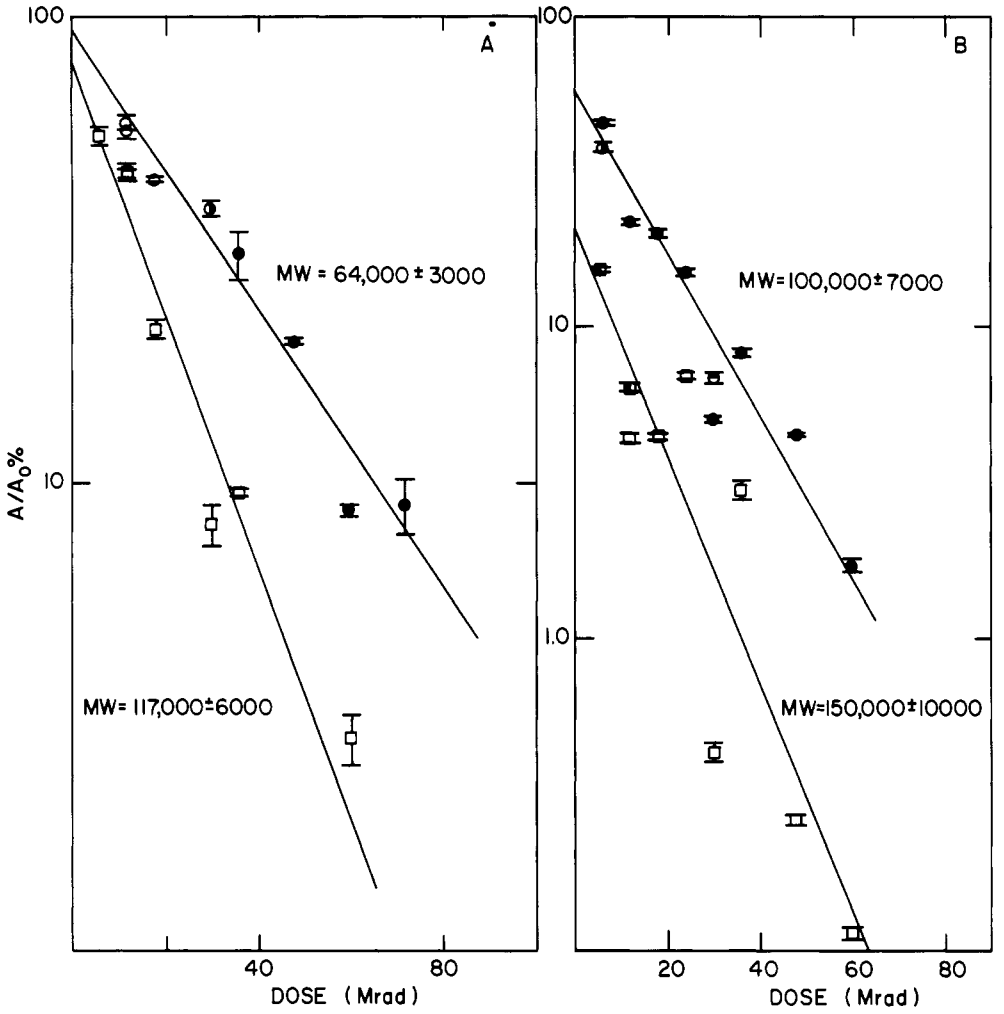

FIGURE 3: (A) Plot of decay in activity of yeast alcohol dehydrogenase irradiated at −135 °C in the presence (○, ◑, ●) and absence (□) of 10 mM thioglycerol. Thioglycerol (10 mM) was also added to the assay buffer in all cases and in some cases to the irradiated samples as a stabilizing agent during thawing and dilution to yield the following final concentrations: no thioglycerol (□); 21 mM in thawing sample, 11 mM in diluted sample (○); 113 mM in thawing sample, 59 mM in diluted sample (◑); 113 mM in thawing sample, 115 mM in diluted sample (●). Each point represents the maximal velocity obtained from the kinetic analysis of multiple assays of a single sample, performed as described under Experimental Procedures. All samples contained 1 mg/mL yeast alcohol dehydrogenase and 1 mg/mL BSA. Assay conditions were the same as those given in the legend of Figure 1 (except that 0.1% BSA was added to the diluted sample and assay buffer instead of gelatin). The lines drawn were obtained from an unconstrained least-squares analysis of the data. (B) Plot of surviving activity of yeast alcohol dehydrogenase after irradiation at −135 °C in the presence (●, ◓, ◑) and absence (□, ◧) of 2 mM dithiothreitol. Thioglycerol (10 mM) was added to the assay buffer in all cases. Dithiothreitol or thioglycerol was added to the irradiated samples to the following final concentrations: 50 mM thioglycerol in thawing and diluted samples (●); 20 mM DTT in thawing and diluted samples (◑); 50 mM DTT and 6 mM ascorbate in thawing and diluted samples (◓); 25 mM DTT in thawing and diluted samples (◧); 50 mM thioglycerol in thawing and diluted samples (□). Each point represents the maximal velocity obtained from the kinetic analysis of a single sample, as in (A). Assay conditions were the same as those described in (A) (except for the use of pH 5.4 in the dilution buffer). The lines drawn were obtained from unconstrained least-squares analysis.

Another set of experiments was designed to test whether the presence of ascorbate in addition to thioglycerol during or after irradiation would provide more effective protection or repair, since oxidized sulfhydryl species were likely products of radical attack (Garrison, 1986). However, no further increase in activity or stability of the enzyme was observed in the presence of ascorbate (data not shown).

Radiation Inactivation Behavior of Horse Liver Alcohol Dehydrogenase. The simpler structure and greater stability of horse liver alcohol dehydrogenase, compared to the closely related yeast enzyme (Branden et al., 1975; Klinman, 1981), suggested that the horse protein might give more reliable data in radiation inactivation analysis and provide more conclusive evidence regarding the subunit requirement for activity.

The horse liver enzyme was irradiated in sealed vials in the presence and absence of thioglycerol. Some time-dependent loss of activity was observed after thawing and dilution of both the control and irradiated samples, but instability was not greatly alleviated by addition of thioglycerol, desferal (an iron chelator), or BHT (butylated hydroxytoluene, a radical scavenger). Thioglycerol was inhibitory to the horse liver alcohol dehydrogenase (HLADH) at levels above 10 mM in the dilution buffer and therefore could not be used at the high concentrations previously found to be necessary to stabilize the yeast protein. However, if desferal and BHT were added together to the dilution buffer, they stabilized the activity sufficiently to permit kinetic analysis to be performed.

A modification of the commonly used Vallee and Hoch (1955) assay procedure was necessary to obtain maximal activities with the horse enzyme, since the high levels of ethanol used in the yeast enzyme assay are inhibitory to the horse protein (Shore & Theorell, 1966). Under the buffer conditions employed, it was also found that NAD concentrations greater than 0.08 mM produced nonlinear Lineweaver–Burk plots (Figure 4) as a result of apparent activation of the enzyme at higher NADH levels. This prevented extrapolation to a

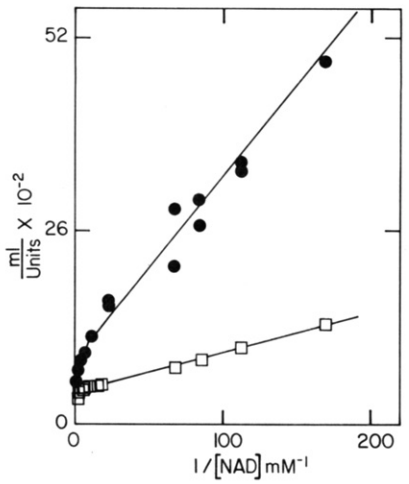

FIGURE 4: Comparison of Lineweaver–Burk plot of kinetics of reduction of NAD by unirradiated (□) and irradiated (●) horse liver alcohol dehydrogenase. The irradiated sample received a dose of 12 Mrad at −135 °C. Activity was measured by the method of Vallee and Hoch (1955) with the following modifications: the assay buffer contained 8.8 mM ethanol, 0.1% BSA was added to the dilution and assay buffers instead of gelatin, and the pH of the dilution buffer was 5.4.

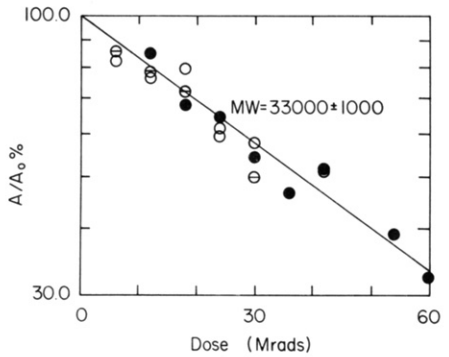

FIGURE 5: Plot of decay in activity of horse liver alcohol dehydrogenase irradiated at −135 °C in the presence (○, ⊖) and absence (●) of 10 mM thioglycerol. In all cases, 1 μM desferal and 0.008% BHT were added to the thawing and diluted samples. Each point represents the highest activity measured for a single sample. All samples contained 7 mg/mL horse liver alcohol dehydrogenase and 1 mg/mL BSA. Activity was measured by the method of Vallee and Hoch (1955), using 32 mM potassium pyrophosphate, 7.7 mM ethanol, 2.7 mM NAD, and 0.1% BSA, pH 8.8. The kinetic analysis of each sample showed consistent K_m values over the lower NADH concentration range for which a linear double-reciprocal plot was obtained (Figure 4).

unique maximal velocity. For this reason the highest rate observed, rather than the extrapolated V_{max}, was used as a measure of the activity remaining in each sample.

As shown in Figure 5, horse liver alcohol dehydrogenase activity exhibits an exponential decay with increasing radiation dose and the curve extrapolates close to 100% at zero dose. There is no significant difference between the degrees of inactivation observed in samples irradiated in the presence or absence of thioglycerol, suggesting that the sulfhydryl groups in this enzyme are not as labile to radiolytic products as those in the yeast alcohol dehydrogenase.

From the data shown in Figure 5, a functional molecular weight of 33 000 ± 1000 was obtained for the horse liver enzyme. This value is close to the subunit molecular weight (M_r = 40 000; Jornvall, 1970) and suggests that the functional unit is comprised of only a single subunit and not a dimer as is often assumed (Lo et al., 1982; Lilly et al., 1983).

As a control for the accuracy of the dosimetry and the temperature correction factor used in this analysis, the physical

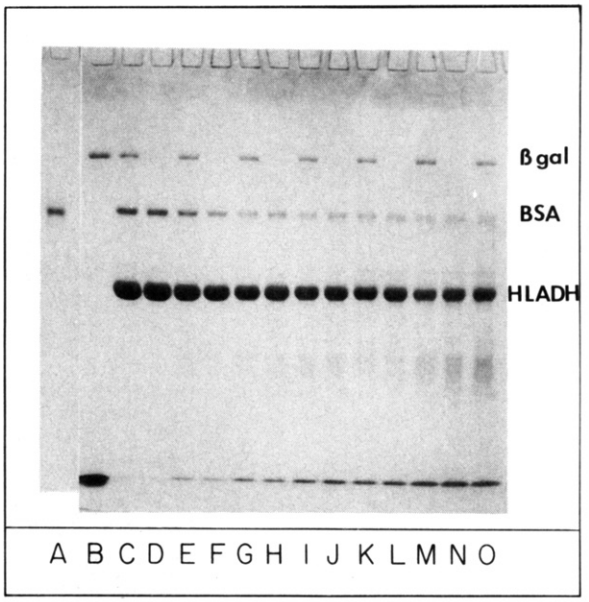

FIGURE 6: SDS–polyacrylamide gel electrophoresis of horse liver alcohol dehydrogenase after various doses of radiation. Gels were loaded with samples containing 70 μg of horse liver alcohol dehydrogenase and 10 μg of bovine serum albumin. Each sample had received the following dose of radiation at −135 °C: C and D, 0 Mrad; E and F, 6 Mrad; G and H, 12 Mrad; I and J, 18 Mrad; K and L, 24 Mrad; M and N, 30 Mrad; O, 36 Mrad. β-Galactosidase (5 μg of Sigma Grade 1X) was added to samples C, E, G, I, K, M, and O to serve as a control for the quantitative loading of the samples into the gel. Lanes A and B were loaded with samples containing only BSA, and β-galactosidase, respectively. Sample and gel preparation and Brilliant Blue staining were performed as described under Experimental Procedures.

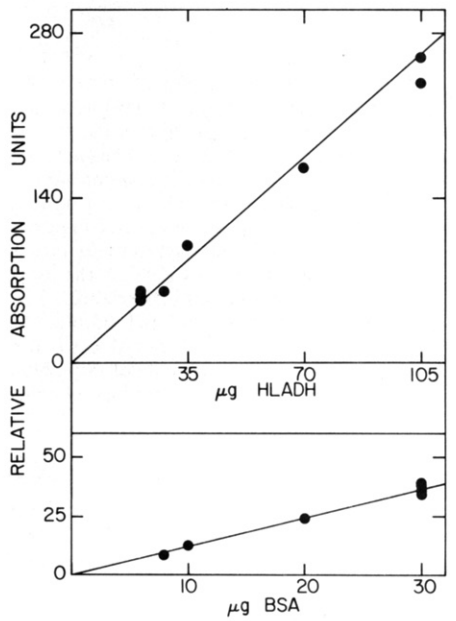

FIGURE 7: Calibration curve of Brilliant Blue staining intensity for horse liver alcohol dehydrogenase (upper panel) and bovine serum albumin (lower panel). Samples containing varying amounts of horse liver alcohol dehydrogenase and bovine serum albumin were loaded on SDS gels, electrophoresed, and stained under the same conditions as those used for irradiated samples. Details of sample and gel preparation and Brilliant Blue staining are given under Experimental Procedures. Scanning of the gels was performed on a Gelman Model ACD-18 densitometer at 575 nm.

destruction of the protein was quantitated by measuring the decrease in staining of the protein band in an SDS gel as a function of dose. Figure 6 shows the disappearance of the horse liver alcohol dehydrogenase and BSA protein bands with

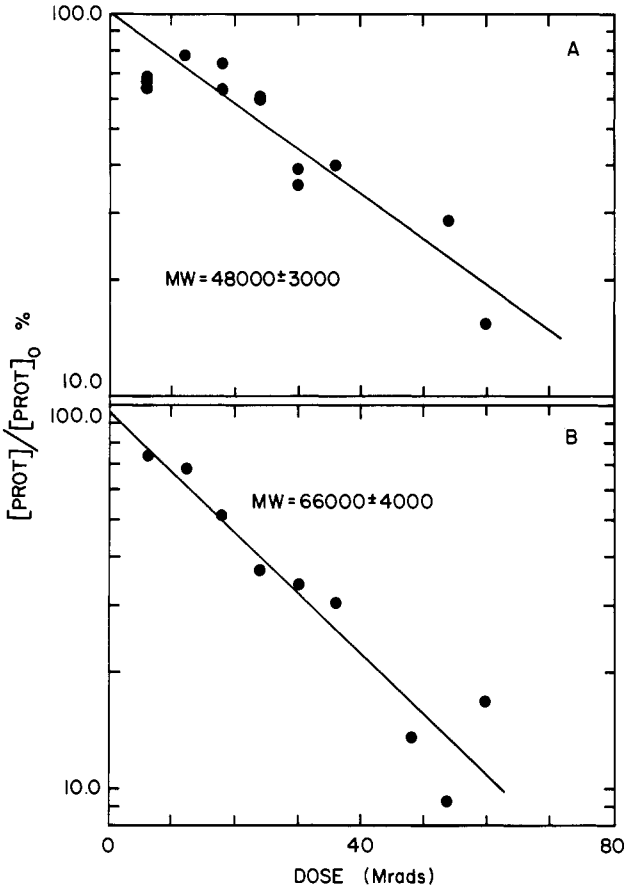

FIGURE 8: Plots of decrease in amount of horse liver alcohol dehydrogenase (panel A) and bovine serum albumin (panel B) with increasing doses of radiation at −135 °C. The amount of protein [Prot] remaining as a function of dose was determined as described in Figure 7 by quantitation of the Brilliant Blue staining of the third (HLADH; M_r = 40 000) and second (BSA; M_r = 68 000) major protein bands from the SDS gel shown in Figure 6. The zero-dose concentration $[Prot]_0$ of HLADH was 70 μg, and the zero-dose concentration of BSA was 20 μg, both within the linear range of the calibration curves shown in Figure 7.

increasing dose and the concomitant appearance of lower molecular weight products. The correspondence between intensity of staining with Brilliant Blue and amount of protein applied to the gel was established for both the alcohol dehydrogenase and BSA in a standard curve (Figure 7). From a plot of the amount of horse liver alcohol dehydrogenase protein remaining vs. dose (Figure 8A), an estimate of 48 000 ± 3000 is obtained, in reasonable agreement with the known molecular weight (40 000) and the size of the functional unit of the enzyme estimated from loss of activity (Figure 5).

As an additional control, the target molecular weight of BSA, which is present in the dehydrogenase samples to promote stability, was also determined from the gels. Consistent with the known molecular weight of the protein (M_r = 68 000) a target size of 66 000 ± 4000 was obtained (Figure 8B).

Discussion

One of the challenging problems in the study of oligomeric enzymes is to distinguish between fortuitous physical aggregation in the purified state and functionally important associations of subunits. The technique of radiation inactivation or target analysis provides a method of measuring the functional rather than the physical size of enzymes and thus offers the possibility of directly addressing this issue. In recent years, the method has been used increasingly to study enzymes and membrane receptors, resulting in estimates of functional size that in many cases are in good agreement with available structure/function information (Kempner & Schlegel, 1979; Kempner & Haighler, 1982; Suarez, 1986; Kempner, 1987). However, a number of discrepancies and inconsistencies have also been observed (Levinson & Ellory, 1974; Parkinson & Callingham, 1982; Ottolenghi & Ellory, 1983; Karlish & Kempner, 1984; McIntyre & Churchill, 1985; Suarez, 1986), and the question arises as to whether these anomalous results are due to failure of target theory or to artifacts arising from the experimental procedure.

One potential source of artifacts is the generation of secondary radiation products during irradiation. The nature and lifetime of these radiolytic species, and the extent to which they will cause damage to the protein at the low temperatures used for irradiation, will be highly dependent on the precise conditions of the experiment and the biochemical properties of the protein. In some cases (but not all) their effects may be revealed by anomalous radiation inactivation plots that extrapolate to a zero-dose intercept that is significantly less than 100% of the original activity, as exemplified by the studies on yeast alcohol dehydrogenase described in this paper. Similar data have been reported for phlorizin-binding protein (Turner & Kempner, 1982). In both cases, an exponential decay of activity as a function of dose was observed, but analysis of the yeast alcohol dehydrogenase data by normalizing to the lower zero-dose activity gave a significantly higher molecular weight estimate than that derived from experiments carried out in the presence of protective thiol reagents. This finding suggests that even though a linear inactivation plot was obtained, damage from secondary radiation products was contributing to the loss of activity over the entire dose range. An obvious concern raised by these data is that even when inactivation plots appear linear, there may be loss of activity due to secondary radiation damage that is not easily discernible.

At the low temperatures used for irradiation it might be expected that the radical species produced in the solvent would be trapped in the crystal lattice and have limited access to the protein. However, the inactivating effect of irradiated water on the yeast enzyme demonstrates that some damaging species can survive at low temperatures for several weeks. These products have not been identified, but possible candidates are H_2O_2 (Durchschlag & Zipper, 1984) or radicals generated from organic contaminants of distilled water. Nevertheless, it is clear that the levels of radiolytic products accumulated under most experimental conditions used for target analysis are not sufficient to cause significant damage to most proteins; only a few, such as yeast alcohol dehydrogenase, phlorizin-binding protein (Turner & Kempner, 1982), and papain (Fluke, 1972), show extraordinary sensitivity. This phenomenon of excessive sensitivity to radiation damage has been attributed to the presence of unusually reactive sulfhydryls that are involved in substrate binding (Armstrong & Buchanan, 1978; Garrison, 1986). When exposed to radiation in the presence of oxygen, these sulfhydryls can be converted to sulfinic and sulfonic derivatives that are not repairable. Thus the extent of damage will be dependent on oxygen concentration, a variable that is not easily controlled, as well as protein concentration and the presence of natural scavengers and other components in the sample. This may explain the wide range of functional molecular weights obtained for yeast alcohol dehydrogenase (Hutchinson et al., 1957; Shikita & Hatano-Sato, 1972; Venter et al., 1983; McIntyre & Churchill, 1985). Indeed, the smallest target size reported (37 000) was determined with dried yeast cells, a system that is likely to provide

a high level of protection from secondary radiation damage. Although the use of dried or lyophilized samples is likely to minimize the formation of damaging radiolytic products, other problems related to protein stability and resolubilization after irradiation often outweigh this advantage (Braams et al., 1958; Lai et al., 1984; Leigh et al., 1984; Karlish & Kempner 1984).

In the presence of high levels of thiol reagents, we find a functional molecular weight of 67 000 ± 3000 for yeast alcohol dehydrogenase, a value close to that of a dimer (M_r = 74 000), but we cannot conclude that this is the minimal functional unit since secondary radiation damage may still be a factor. The zero-dose intercept is close to 100% and a linear semilog plot is obtained, but increasing dose can produce increasing levels of radiolytic products that may make a dose-dependent contribution to the inactivation of the enzyme. This possibility must also be considered when dealing with other proteins that may have less sensitivity to secondary radiation damage, so that the effect is not seen at the lower dose levels, giving rise to an inactivation plot with a normal zero-dose intercept but an artifactually steep slope.

Horse liver alcohol dehydrogenase has an active-site configuration very similar to that of the yeast enzyme, and the main features of their catalytic mechanisms appear to be the same (Branden et al., 1975; Klinman, 1981). However, the sulfhydryl groups that are essential for activity are less chemically reactive than those in the yeast protein (Reynolds & McKinley-McKee, 1975), and the two zinc atoms are more stably bound. Unlike the yeast enzyme, target analysis of the horse alcohol dehydrogenase resulted in a normal inactivation plot with an exponential dependency on dose and a zero-dose intercept close to 100%. The functional size estimate, 33 000 ± 1000, was the same in the presence or absence of thiol reagents, indicating that this protein is much less sensitive to damaging effects of radiolytic products. Quantitative analysis of the disappearance of the protein band from the SDS gel of the irradiated enzyme gave a molecular weight estimate of 48 000 ± 3000, in reasonable agreement with the known subunit molecular weight of 40 000 (Jornvall, 1970). This finding, together with the accurate estimate of the size of BSA by the same method (target size 66 000 ± 4000; M_r = 68 000, from known sequence), validates the dosimetry and the temperature correction factor used in these analyses and gives strength to the conclusion that a single subunit of horse liver alcohol dehydrogenase is the minimal functional unit.

Previous kinetic studies (Bernhard et al., 1970; McFarland & Bernhard, 1972; Gerschitz et al., 1978; Dunn et al., 1979) and structural information (Eklund et al., 1976) have suggested that subunit interactions in the dimer form are involved in the catalytic function of horse liver alcohol dehydrogenase. However, in agreement with our target analysis results, more recent transient kinetic studies demonstrate that the biphasic behavior previously interpreted to result from interactions between subunits is exhibited by a single catalytic site (Anderson & Dahlquist, 1982; Anderson et al., 1982). Given the functional similarity of the yeast and horse alcohol dehydrogenases, the implication of these findings is that a single subunit of the yeast enzyme may also be independently active.

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Optically Detected Magnetic Resonance of Tryptophan Residues in Complexes Formed between a Bacterial Single-Stranded DNA Binding Protein and Heavy Atom Modified Poly(uridylic acid)†

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ABSTRACT: Optically detected magnetic resonance (ODMR) methods were employed to study three single-stranded DNA binding (SSB) proteins encoded by plasmids of enteric bacteria: pIP71a, R64, and F. Equilibrium binding isotherms obtained by fluorescence titrations reveal that the complexes of the plasmid SSB proteins with heavy atom modified polynucleotides are readily disrupted by salt. Since all the plasmid SSB proteins show limited solubility at low ionic strength (pIP71a > R64 > F), we were able to bind only the pIP71a protein to mercurated poly(uridylic acid) [poly(5-HgU)] and brominated poly(uridylic acid) [poly(5-BrU)]. ODMR results reveal the existence of at least one heavy atom perturbed, red-shifted, stacked Trp residue in these complexes. Amplitude-modulated phosphorescence microwave double resonance spectra display selectively the phosphorescence associated with Hg-perturbed Trp residue(s) in the pIP71a SSB protein–poly(5-HgU) complex, which has a broad, red-shifted 0,0-band. Our results suggest that Trp-135 in *Escherichia coli* SSB, which is absent in the plasmid-encoded SSB proteins, is located in a polar environment and is not involved in stacking interactions with the nucleotide bases. Phosphorescence spectra and lifetime measurements of the pIP71a SSB protein–poly(5-BrU) complex show that at least one Trp residue in the complex does not undergo stacking. This sets a higher limit of two stacking interactions of Trp residues with nucleotide bases in complexes of pIP71a SSB with single-stranded polynucleotides.

Single-stranded DNA (ssDNA)[1] binding proteins (SSB proteins) play a vital role in cellular processes such as DNA replication, recombination, and repair. SSB proteins that bind preferentially to single-stranded DNA and seem devoid of catalytic activity have been isolated from a variety of sources and organisms, both prokaryotic and eukaryotic. The physiological functions of these proteins have been best characterized from phage T4 and *Escherichia coli* [for reviews, see Falaschi et al. (1980), Kowalczykowsky et al. (1981), Williams and Konigsberg (1981), Hélène et al. (1982), and Chase and Williams (1986)]. Genetic analysis of conditional lethal mutants of *E. coli* single-strand binding protein (Eco SSB) has established its necessary role in DNA replication, repair, and recombination (Meyer et al., 1979; Whittier & Chase,

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[1] Abbreviations: AM-PMDR, amplitude-modulated phosphorescence microwave double resonance; D and E, triplet-state zero-field splitting parameters; dsDNA, double-stranded DNA; EDTA, ethylenediaminetetraacetic acid; Lys-Trp-Lys, lysyltryptophyl-α-lysine; ODMR, optically detected magnetic resonance; pIP71a SSB, single-stranded DNA binding protein encoded by pIP71a, an Inc9 plasmid; pIP231a SSB, single-stranded DNA binding protein encoded by pIP231a, an IncY plasmid; R64 SSB, single-stranded DNA binding protein encoded by R64, an $IncI_1$ plasmid; *E. coli* SSB (*Eco* SSB), single-stranded DNA binding protein encoded by *Escherichia coli*; F plasmid SSB (F SSB), *E. coli* F plasmid encoded single-stranded binding protein; poly(dT), poly(deoxythymidylic acid); poly(5-BrU), brominated poly(uridylic acid); poly(5-HgU), mercurated poly(uridylic acid); ssDNA, single-stranded (heat-denatured) DNA; Trp, tryptophan.

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