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Rate-Determining Folding and Association Reactions on the Reconstitution Pathway of Porcine Skeletal Muscle Lactic Dehydrogenase after Denaturation by Guanidine Hydrochloride†

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ABSTRACT: Reactivation of tetrameric porcine skeletal muscle lactic dehydrogenase after dissociation and extensive unfolding of the monomers by 6 M guanidine hydrochloride (Gdn·HCl) is characterized by sigmoidal kinetics, indicating a complex mechanism involving rate-limiting folding and association steps. For analysis of the association reactions, chemical cross-linking with glutaraldehyde may be used [Hermann, R., Jaenicke, R., & Rudolph, R. (1981) *Biochemistry 20*, 2195-2201]. The data clearly show that the formation of a dimeric intermediate is determined by a first-order folding reaction of the monomers with $k_1 = (8.0 \pm 0.1) \times 10^{-4}$ s^{-1}. The rate constant of the association of dimers to tetramers, which represents the second rate-limiting step on the pathway of reconstitution after guanidine denaturation, was then determined by reactivation and cross-linking experiments after dissociation in 0.1 M H_3PO_4 containing 1 M Na_2SO_4. The rate constant for the dimer association (which is the only rate-limiting step after acid dissociation) was $k_2 = (3.0 \pm 0.5) \times 10^4$ M^{-1} s^{-1}. On the basis of the given two rate constants, the complete reassociation pattern of porcine lactic dehydrogenase after dissociation and denaturation in 6 M Gdn·HCl can be described by the kinetic model

$$4M \xrightarrow{k_1} 4M^* \xrightarrow{\text{fast}} 2D \xrightarrow{k_2} T$$

Reconstitution experiments with oligomeric enzymes have been frequently performed in the past in order to make clear various aspects of the subunit interaction such as the correlation of quaternary structure and biological activity (Jaenicke & Rudolph, 1980). Rate measurements of the reactivation of numerous enzymes after previous denaturation and dissociation often revealed sigmoidal kinetics (Vallee & Williams, 1975; Rudolph et al., 1977a,b; Jaenicke et al., 1979; Zabori et al., 1980; Dautry-Varsat & Garel, 1978), indicating a complex reconstitution mechanism with rate-limiting folding and association steps. Although little information was available regarding the sequence of events and the nature of the intermediate states, the observed sigmoidal kinetics were previously ascribed to a model consisting of two consecutive

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0006-2960/82/0421-3946$01.25/0

first-order and second-order reactions (Rudolph et al., 1977a,b; Jaenicke et al., 1979; Zabori et al., 1980).

Recently we have developed a method to analyze individual steps in complex association reactions of oligomeric proteins (Hermann et al., 1979, 1981). The approach is based on fast chemical cross-linking during reassociation. The distribution of molecular species at any time during the process of reconstitution is quantified by $NaDodSO_4$[1]–polyacrylamide gel electrophoresis of the cross-linked material. The method allows the determination of the rate constants of individual association steps and the identification of intermediates of association.

With the use of this approach the present study reveals the complete sequence of events that are rate determining for the reassociation of tetrameric pig skeletal muscle lactic dehydrogenase after dissociation and maximum unfolding by 6 M guanidine hydrochloride.

Materials and Methods

Substances. Lactic dehydrogenase (EC 1.1.1.27; lactate dehydrogenase) from pig skeletal muscle was obtained from Boehringer (Mannheim), and glutaraldehyde [purissimum, 25% or 50% (w/v) aqueous solution] was from Fluka (Basel). All other reagents were the same as described elsewhere (Zettlmeissl et al., 1981).

Standard buffer was 0.1 M phosphate buffer, pH 7.6, containing 1 mM EDTA, degassed and saturated with nitrogen. The preparation of stock solutions of the enzyme, concentration determinations, and enzymatic tests were performed as described previously (Zettlmeissl et al., 1981).

Denaturation/Reconstitution Procedures. Denaturation of the native enzyme by Gdn·HCl was achieved by 1:5 dilution with 7.5 M Gdn·HCl in standard buffer plus 1 mM dithioerythritol, leading to a final denaturant concentration of 6 M (pH 6.0 ± 0.1); the incubation time was 5 min. Acid dissociation of the native enzyme was performed by 15-min incubation at 20 °C, after 1:10 dilution in 0.1 M H_3PO_4 containing 1 M Na_2SO_4 [for experimental details, see Hermann et al. (1981)]. Reconstitution was initiated by 1:500 dilution with standard buffer at 20 °C. During reconstitution, after acid dissociation, 0.012 M Gdn·HCl was added to the reactivation buffer so that the Gdn·HCl content during reconstitution was identical after both guanidine denaturation and acid dissociation.

The reactivation data after guanidine denaturation, which are calculated relative to the final reactivation (45 ± 10% after 72 h), are referred to as "relative reactivation". After acid dissociation, reactivation amounts to 70 ± 10% after 72 h. For these experiments the reactivation data, referred to as "reactivation", are calculated relative to the total amount of denatured enzyme.

Cross-Linking during Reassociation. The kinetics of reassociation were analyzed by a modified version of the cross-linking procedure described earlier by Hermann et al. (1979, 1981). In the present study 0.012 M Gdn·HCl was present during the glutaraldehyde fixation, due to the reconstitution from 6 M Gdn·HCl. The residual denaturant does not interfere with the cross-linking: experiments using native lactic dehydrogenase show that the enzyme can be cross-linked almost quantitatively in the presence of up to 0.03 M Gdn·HCl. After a 2-min incubation with glutaraldehyde, dissociation products or higher aggregates, caused by either incomplete fixation or intermolecular cross-linking of the native tetramers, do not exceed 5% on the $NaDodSO_4$–polyacrylamide gels (data not shown).

[1] Abbreviations: EDTA, (ethylenedinitrilo)tetraacetic acid; k_1 and k_2, rate constants; $NaDodSO_4$, sodium dodecyl sulfate; Gdn·HCl, guanidine hydrochloride.

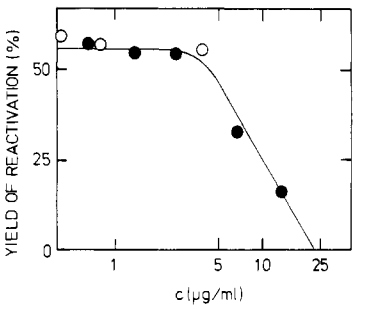

FIGURE 1: Effect of enzyme concentration on the yield of reactivation of pig muscle lactic dehydrogenase after 5-min denaturation in 6 M Gdn·HCl at 0 °C (○) and 20 °C (●). Reactivation was determined after 72 h at 20 °C in standard buffer plus 1 mM dithioerythritol and 0.03 M Gdn·HCl (residual concentration).

The high molecular weight aggregates, which form rapidly during reconstitution (Zettlmeissl et al., 1979b, 1982), are only partially cross-linked. Some of the aggregated polypeptide chains—presumably from the interior of the aggregates—are released as monomers and dimers upon $NaDodSO_4$ treatment.

The amount of this material was determined by cross-linking after 24 h of reconstitution, and the association data were corrected accordingly. For the reassociation analysis 35-mL aliquots of reassociating lactic dehydrogenase were cross-linked by rapid addition of 1% (w/v) glutaraldehyde. The cross-linking reaction was stopped after 2 min by a ~10-fold excess of solid $NaBH_4$. From this solution the cross-linked protein was precipitated after 5 min by a combined sodium deoxycholate/trichloroacetic acid precipitation (Bensadoun & Weinstein, 1976). Precipitation was performed by adding 0.05–0.1 mL of 10% (w/v) aqueous sodium deoxycholate, followed by careful addition of 3.5–6 mL of trichloroacetic acid (78% w/v) in order to destroy excess $NaBH_4$ and to achieve precipitation. The precipitated material was separated by centrifugation (30 min, 4000*g*) and redissolved in a small volume of water (0.2 mL) containing 10% $NaDodSO_4$ (w/v) and 50 mM dithioerythritol plus 0.02 mL of concentrated sodium hydroxide. The precipitation step substitutes for the tedious and time-consuming dialysis against 0.2% (w/v) $NaDodSO_4$ and subsequent lyophilization, which was previously used to remove excess reagent and to increase the protein concentration (Hermann et al., 1981). The sensitivity of the cross-linking procedure is increased considerably by the precipitation step, so that association studies by cross-linking can be performed at enzyme concentrations as low as 0.4 µg/mL. So that artifacts due to cystine formation could be avoided, samples were heated (10 min at 100 °C) in the presence of dithioerythritol. The cross-linked products were separated by $NaDodSO_4$–polyacrylamide gel electrophoresis as described elsewhere (Hermann et al., 1981). The cross-linked aggregates were separated from monomers and cross-linked dimers, as well as tetramers, as a diffuse band in the upper gel with a large pore size.

Results

Reactivation and Reassociation after Gdn·HCl Denaturation. The maximum yield of reactivation of porcine muscle lactic dehydrogenase after denaturation with 6 M Gdn·HCl amounts to 45 ± 10%. This value remains constant in the concentration range from 0.4 to ~5 µg/mL (Figure 1). At concentrations >5 µg/mL the amount of reactivated enzyme decreases due to an increase in the rate of unspecific aggregation (Zettlmeissl et al., 1979b, 1982).

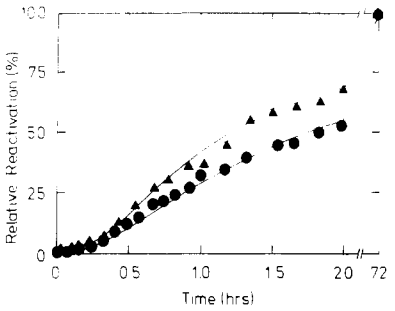

FIGURE 2: Kinetics of reactivation of porcine skeletal muscle lactic dehydrogenase after denaturation in 6 M Gdn·HCl at 20 °C. Reactivation was at 20 °C by dilution in standard buffer [residual guanidine concentration was 0.012 M; concentration of reactivated enzyme was 1 (▲) and 0.55 μg/mL (●)]. Solid lines are calculated according to an irreversible first-order/second-order mechanism (eq 1) with $k_1 = 8 \times 10^{-4}$ s^{-1} and $k_2 = 3 \times 10^4$ M^{-1} s^{-1}.

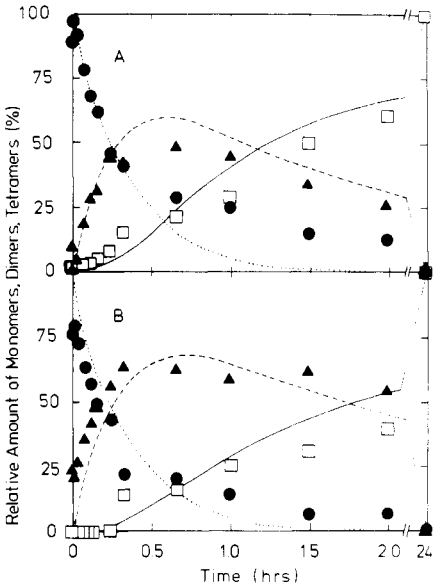

FIGURE 3: Kinetics of reassociation of pig muscle lactic dehydrogenase after denaturation in 6 M Gdn·HCl. Reassociation (20 °C) at a concentration of reactivated enzyme of 1 (A) and 0.55 μg/mL (B) was determined by cross-linking with glutaraldehyde and subsequent NaDodSO$_4$–polyacrylamide gel electrophoresis. The relative amounts of monomers (●), dimers (▲), and tetramers (□) were determined by densitometry after electrophoresis. The data are corrected for approximately 8% monomers and 22% dimers still present after 24 h of reassociation. These particles most probably stem from the dissociation of partially cross-linked higher aggregates by NaDodSO$_4$. The solid lines are calculated according to eq 1 with the rate constants given in Figure 2.

The kinetics of reactivation after complete unfolding by Gdn·HCl are characterized by sigmoidal relaxations (Figure 2), indicating a consecutive reaction sequence with at least one rate-limiting step in addition to the slow dimer → tetramer association determining the reconstitution after acid denaturation (Rudolph & Jaenicke, 1976).

The reassembly of the enzyme has been quantitatively analyzed by chemical cross-linking with glutaraldehyde as a bifunctional reagent. As shown for the two isoenzymes of lactic dehydrogenase, the reagent fulfills all the necessary requirements in order to be applied in studies on protein self-association: (i) intramolecular (intersubunit) cross-linking of dimeric intermediates and tetramers is quantitative; (ii) no intermolecular cross-links between free particles are formed; (iii) cross-linking is fast under appropriate experimental conditions (Hermann et al., 1981; Bernhardt et al., 1981). For the reassociation of the isoenzyme from pig skeletal muscle

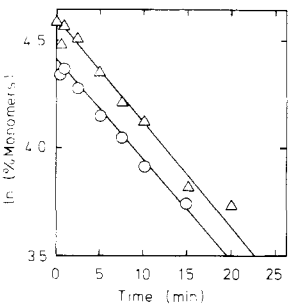

FIGURE 4: First-order linearization of the decrease in monomer concentration during reassociation of lactic dehydrogenase as shown in Figure 3. The following rate constants were determined: 1 μg/mL (△), 8.1×10^{-4} s^{-1}; 0.55 μg/mL (○), 7.9×10^{-4} s^{-1}.

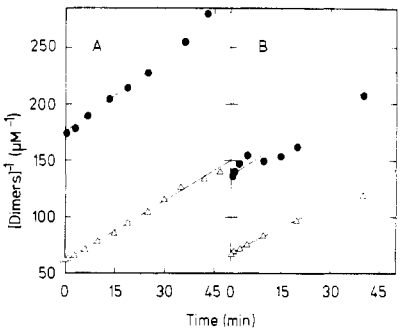

FIGURE 5: Second-order linearization of reactivation (A) and tetramer formation (B) of lactic dehydrogenase after acid dissociation in the presence of 1 M Na_2SO_4. Reconstitution by dilution in standard buffer plus 0.012 M Gdn·HCl. The following rate constants were determined for the respective concentrations of reactivated enzyme: (A) for 1.1 μg/mL, 3.0×10^4 M^{-1} s^{-1} (△), and for 0.40 μg/mL, 3.5×10^4 M^{-1} s^{-1} (●); (B) for 1.1 μg/mL, 2.5×10^4 M^{-1} s^{-1} (△), and for 0.4 μg/mL, 3.0×10^4 M^{-1} s^{-1} (●). Rate constants refer to the dimer concentration.

the quantitative densitometric analysis clearly shows that only dimers are populated as intermediates of reconstitution (Figure 3). The decrease in monomer concentration obeys first-order kinetics with a rate constant $k_1 = (8.0 \pm 0.1) \times 10^{-4}$ s^{-1} (Figure 4). It is paralleled by the accumulation of dimers. This result allows the conclusion that the rate-limiting step in the formation of dimeric intermediates is a first-order folding reaction of the denatured monomers. The second rate-limiting step on the pathway of reconstitution consists of the association of dimers to tetramers, as shown by the concentration dependence of the rate of tetramer formation. Tetramerization follows sigmoidal kinetics that are governed by a consecutive reaction sequence, determined by first-order folding of monomers and second-order association of dimers. Having determined the first-order rate constant of the monomer folding, we measured the rate constant of the second-order dimer → tetramer association directly by reconstitution experiments after acid dissociation.

Reactivation and Reassociation after Acid Dissociation. The only rate-limiting step on the pathway of reconstitution of the acid-dissociated monomers of porcine muscle lactic dehydrogenase is the association of dimers to tetramers (Hermann et al., 1981). The rate of this second-order reaction was found to be strongly affected by low concentrations of Gdn·HCl present during reconstitution: previous binding studies have shown that guanidine binding to intermediates of reconstitution yields a significant deceleration of the rate of reactivation (Zettlmeissl et al., 1979a). Thus the amount of guanidine present during reactivation after Gdn·HCl denaturation had to be added to the reconstitution buffer after acid dissociation, in order to obtain the same rate constant for the dimer to tetramer association in both processes. As shown

in Figure 5A, reactivation after acid dissociation follows the previously described second-order kinetics. Tetramer formation, as determined by chemical cross-linking, strictly parallels reactivation (Figure 5B). In the presence of 0.012 M Gdn·HCl a second-order rate constant of $k_2 = (3.0 \pm 0.5) \times 10^4$ M^{-1} s^{-1} was determined for both reactivation and dimer → tetramer association.

Reconstitution Mechanism after Guanidine Denaturation. The two previously determined rate constants of the first-order monomer folding (k_1) and of the dimer to tetramer association (k_2) may be used to quantitatively describe the observed reconstitution pattern of pig muscle lactic dehydrogenase after dissociation and denaturation by 6 M Gdn·HCl. The most simple kinetic model to summarize the reactivation data would be the consecutive first-order/second-order mechanism

$$4M \xrightarrow{k_1} 4M^* \xrightarrow{\text{fast}} 2D \xrightarrow{k_2} T \qquad (1)$$

In this model M and M* stand for unfolded and folded monomers, respectively, while D and T represent the dimeric intermediate and the native tetramer. The complete solution in closed form for a consecutive first-order/second-order reaction of this type has been previously presented by Chien (1948). As shown in Figure 3, this model gives an almost quantitative description of the experimentally determined monomer, dimer, and tetramer population in the process of reconstitution of lactic dehydrogenase.

Discussion

Reconstitution of porcine skeletal muscle lactic dehydrogenase has been studied in the past in considerable detail in order to elucidate various aspects of the self-association of oligomeric enzymes (Jaenicke & Rudolph, 1980). In the course of this work, reactivation was studied after denaturation by acid (Rudolph & Jaenicke, 1976) or by high hydrostatic pressure (Schade et al., 1980), and the mechanism of the competition between reactivation and the formation of inactive aggregates was analyzed (Zettlmeissl et al., 1979b). Reactivation of partially folded monomers of the isoenzyme from muscle after brief acid dissociation was found to follow second-order kinetics (Rudolph & Jaenicke, 1976).

Many experimental approaches were applied to define the rate-limiting association step in the overall reconstitution. These methods include (i) fast chemical cross-linking of intermediates of association by glutaraldehyde and subsequent population analysis by $NaDodSO_4$–polyacrylamide gel electrophoresis (Hermann et al., 1979, 1981), (ii) quenching of the association process by limited proteolysis with thermolysin (Girg et al., 1981), or (iii) reassociation studies starting from dimeric intermediates of dissociation produced by ~2 M urea or by 0.8–1.2 M Gdn·HCl (Jaenicke et al., 1981). In the course of these studies the dimer to tetramer association was identified unambigously as the rate-limiting reaction observed upon reactivation after acid dissociation. A decrease in both the rate and the yield of reactivation upon long-term acid incubation has been shown to be caused by a conformational rearrangement within the partially folded monomers (Zettlmeissl et al., 1981). This reaction can be prevented by adding 1 M Na_2SO_4 during dissociation, thus stabilizing elements of residual native backbone structure by reversible precipitation of the acid-dissociated monomers (Hermann et al., 1981).

Reconstitution after complete unfolding of the enzyme by 6 M Gdn·HCl leads to much lower yields compared to reconstitution after acid dissociation in the presence of Na_2SO_4. Obviously, Gdn·HCl denaturation enhances the formation of incorrect intra- and interchain interactions, which finally lead to "wrong aggregation". Upon structure formation in vivo this side reaction may not occur due to domain folding during protein biosynthesis (Bergman & Kuehl, 1979). This difference between in vivo folding and in vitro folding may be responsible for the complete failure in the reconstitution of certain enzymes (Jaenicke, 1982). The yields of reactivated enzyme after acid denaturation and denaturation in 6 M Gdn·HCl are dominated by the kinetic competition between reconstitution on the one hand and the formation of inactive aggregates on the other. It has been shown that neither process is determined by cis ⇌ trans isomerism of X–Pro peptide bonds (Zettlmeissl et al., 1981, 1982).

Due to differences in the temperature dependence of folding and aggregation, the yield of reactivation increases with decreasing temperature (Zettlmeissl et al., 1982). On the other hand, high enzyme concentrations cause a decrease in yield, due to the increase in the rate of unspecific aggregation [Figure 1; cf. Zettlmeissl et al. (1979b)].

Reactivation of pig skeletal muscle lactic dehydrogenase after denaturation by Gdn·HCl follows sigmoidal kinetics. Similar profiles have been observed for the reactivation of the heart muscle isoenzyme after denaturation by acid, Gdn·HCl, urea, or high hydrostatic pressure (Rudolph et al., 1977a; Müller et al., 1981). The respective relaxations have been described by a consecutive first-order/second-order reaction mechanism, although there was no direct evidence to establish the sequence of events nor the nature of the stable intermediate (Rudolph et al., 1977a). In the present study intermediates on the pathway of reconstitution of skeletal muscle lactic dehydrogenase after dissociation and denaturation by Gdn·HCl were analyzed by chemical cross-linking with glutaraldehyde. This method allows determination of the individual rate constants of the consecutive reactions responsible for the sigmoidal relaxations. The results clearly show that dimers are the only intermediates of association. Their formation is determined by a rate-determining first-order folding reaction of the monomers [$k_1 = (8.0 \pm 0.1) \times 10^{-4}$ s^{-1}], while the assembly of dimers to tetramers represents the second rate-limiting step on the reconstitution pathway. The rate constant of this reaction has been separately determined by cross-linking after acid dissociation [$k_2 = (3.0 \pm 0.5) \times 10^4$ M^{-1} s^{-1}], where association represents the only rate-limiting reconstitution step.

On the basis of the individually determined rate constants of monomer folding and dimer association, the reassociation of pig skeletal muscle lactic dehydrogenase after Gdn·HCl denaturation can be fitted by the consecutive first-order/second-order reaction mechanism given in eq 1 if inactive intermediates are assumed. Deviations of the monomer/dimer ratio during the final stage of reactivation may be caused by a fast monomer ⇌ dimer dissociation–association equilibrium. An equilibrium of this type was previously determined during reconstitution after acid dissociation (Hermann et al., 1981). Thus, the most probable reaction mechanism to describe the reconstitution of lactic dehydrogenase from pig skeletal muscle would be

$$4M \xrightarrow{k_1} 4M^* \overset{\text{fast}}{\rightleftharpoons} 2D \xrightarrow{k_2} T \qquad (2)$$

Due to its mathematical complexity, no attempt was made to further develop the complete solution of this equation.

The observed mechanism of reassociation clearly indicates that in the case of lactic dehydrogenase the tetramer represents the only enzymatically active entity. Catalytic function of monomers and dimers in free solution is incompatible with the present reactivation data, as well as independent experimental results reported earlier (Jaenicke et al., 1981; Girg et al., 1981; Hermann et al., 1981). Contradicting evidence using the

matrix-bound enzyme is controversial (Cho & Swaisgood, 1972, 1974; Chan & Mosbach, 1976; Ashmarina et al., 1981). Whether the matrix in this case "mimics" the subunit interface in the native quaternary structure or whether flexibility of the chain molecules forming the matrix is involved needs to be clarified.

Acknowledgments

Expert help and advice of R. Hermann in performing the cross-linking experiments and helpful discussions with Drs. P. Bartholmes and F. X. Schmid are gratefully acknowledged.

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N-Hydroxysulfosuccinimide Active Esters: Bis(*N*-hydroxysulfosuccinimide) Esters of Two Dicarboxylic Acids Are Hydrophilic, Membrane-Impermeant, Protein Cross-Linkers†

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ABSTRACT: We have synthesized and characterized *N*-hydroxysulfosuccinimide, a new hydrophilic ligand for the preparation of active esters. We have incorporated this ligand into two new protein cross-linking reagents, 3,3′-dithiobis(sulfosuccinimidyl propionate) and bis(sulfosuccinimidyl) suberate. In experiments with rabbit muscle aldolase, it is demonstrated that both of these reagents are highly efficient protein cross-linkers at physiological pH and that 3,3′-dithiobis(sulfosuccinimidyl propionate) is quantitatively cleavable by reduction under mild conditions. In experiments with intact human erythrocytes and erythrocyte membranes, it is shown that both reagents are membrane impermeant and that when erythrocytes are treated with either reagent, both cross-link subunits of the anion channel (band 3) to covalent dimers at the extracytoplasmic membrane face.

We have recently demonstrated the utility of a membrane-impermeant, cleavable cross-linker for probing near-est-neighbor relationships of membrane protein subunits at one face of a biological membrane (Staros et al., 1981). The reagent that we introduced for this purpose, diisethionyl 3,3′-dithiobis(propionimidate), is a bis(alkylimidate) and is a membrane-impermeant analogue of dimethyl 3,3′-dithiobis(propionimidate), introduced by Wang & Richards (1974a,b, 1975).

As protein modification reagents, alkylimidates have the distinct advantage that the product amidines are charged at physiological pH like the primary amines from which they are

† From the Department of Biochemistry, Vanderbilt University, School of Medicine, Nashville, Tennessee 37232. *Received March 8, 1982.* This work was supported in part by grants from the National Institutes of Health (AM25489 and RR05424) and in part by a grant from the Petroleum Research Fund, administered by the American Chemical Society (PRF 11139-G1). A preliminary account of some of this work was presented at the Third Biophysical Discussion, Oct 3–6, 1981 (Staros, 1982).

0006-2960/82/0421-3950$01.25/0