α-Aminoaldehydes: Transition State Analogue Inhibitors of Leucine Aminopeptidase†

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ABSTRACT: L-Leucinal, prepared by enzymatic oxidation of L-leucinol with alcohol dehydrogenase, is found to be a very strong competitive inhibitor of porcine kidney aminopeptidases. For the enzyme from kidney microsomes acting on L-leucine *p*-nitroanilide ($K_m = 5.2 \times 10^{-4}$ M), K_i for L-leucinal was 7.6×10^{-7} M at pH 7.2 and 25 °C. For the enzyme from kidney cytosol acting on L-leucine *p*-nitroanilide ($K_m = 7.7 \times 10^{-4}$ M), K_i for L-leucinal was 6×10^{-8} M; K_i for glycinal (analogous to glycine derivatives that are poor substrates) was 6.8×10^{-4} M. In dilute aqueous solution, leucinal exists in unfavorable equilibrium with its covalent hydrate, whose concentration exceeds that of the free aldehyde by a factor of 40. The affinity of the enzyme for the free aldehyde is correspondingly greater than its K_i values would suggest, exceeding the apparent affinity of the substrate by a factor of about 10^6. A comparison of binding affinities suggests that L-leucinal forms an inhibitory complex analogous in structure to unstable intermediates in substrate transformation by leucine aminopeptidase, and strengthens the likelihood that this enzyme may act by a double-displacement mechanism.

Leucine aminopeptidase, an oligomeric enzyme requiring metal ions for activity, catalyzes hydrolysis of proteins, peptides, and amino acid amides. Found in most organisms and cell fractions that have been examined, aminopeptidase activities seem likely to be responsible, at least in part, for the rapid breakdown of numerous proteins and biologically active peptides [for reviews, see Smith & Hill (1960) and Delange & Smith (1971)]. Although their mechanism of action remains unclear, it has been observed that halomethyl ketones, prepared as potential active site directed irreversible inhibitors of leucine aminopeptidase, serve instead as strong *reversible* inhibitors. For the porcine kidney enzyme, a K_i of 1.2×10^{-6} M was observed for leucine chloromethyl ketone, as compared with a K_m somewhat in excess of 10^{-4} M for the substrate leucine *p*-nitroanilide (Birch et al., 1972). Comparable observations were later reported for an enzyme from *Aeromonas proteolytica* (Kettner et al., 1974).

These results, not anticipated according to the hypothesis on which the design of these inhibitors was based, might be explicable in terms of the known susceptibility of halomethyl ketones to reversible addition of nucleophiles (Lewis & Wolfenden, 1977a). Strongly electrophilic carbonyl compounds, structurally analogous to good substrates in other respects, might react reversibly with a nucleophile at the active site of aminopeptidase to form adducts resembling "tetrahedral" intermediates in substrate transformation. Other hydrolytic enzymes including papain (Westerik & Wolfenden, 1972), elastase (Thompson, 1973), and asparaginase (Westerik & Wolfenden, 1974) are inhibited strongly but reversibly by specific aldehydes, which have been shown to form reversible adducts with active-site nucleophiles (Lewis & Wolfenden, 1977b; Clark et al., 1977; Brayer et al., 1979).

If aminopeptidases were to act by mechanisms similar to those of papain or elastase, then it might be predicted that unsubstituted α-aminoaldehydes analogous to good substrates should serve as unusually effective inhibitors. It seemed worthwhile to test this hypothesis.

† From the Department of Biochemistry, University of North Carolina, Chapel Hill, North Carolina 27514. *Received March 16, 1982.* This work was supported by Research Grant GM-18325 from the National Institutes of Health, Institutional Grant 1N-15V from the American Cancer Society (to L.A.), and Training Grant AM-07386 from the National Institutes of Health (to T.C.I.).

Materials and Methods

Materials. Leucine aminopeptidase from porcine kidney cytosol, leucine aminopeptidase from porcine kidney microsomes, L-leucine *p*-nitroanilide, horse liver alcohol dehydrogenase, β-NAD^+, semicarbazide, and FMN (flavin mononucleotide) were purchased from Sigma Chemical Co. Sodium tetradeuterioborate and L-leucine methyl ester hydrochloride were purchased from Alfa Chemical Co. L-Leucinol and aminoacetaldehyde diethyl acetal were obtained from Aldrich Chemical Co. Other chemicals were of analytical grade and were used without further purification. R_f values refer to thin-layer chromatography on Eastman 6060 silica-coated sheets or on Eastman 6065 cellulose-coated sheets. Compounds equipped with primary amino functions were detected by spraying with ninhydrin (0.1% in 1-butanol saturated with water) and heating at 105 °C for 5 min. Aldehydes were visualized after spraying the sheets with a solution containing 2,4-dinitrophenylhydrazine (Johnson, 1951).

Enzyme Preparations. Cytosolic leucine aminopeptidase from pig kidney, obtained as a crystalline suspension from Sigma Chemical Co., was dialyzed twice at 4 °C against a solution (500 mL) containing ammonium sulfate (3 M), 0.1 M triethanolamine hydrochloride (0.1 M, pH 8), and $MgCl_2$ (5×10^{-3} M). The dialyzed enzyme (0.6 mg) was activated for 2 h at 37 °C in a solution (3.45 mL) containing $MnCl_2$ (1×10^{-3} M) and triethanolamine hydrochloride (0.022 M, pH 8.5), and its concentration was estimated assuming $A_{280nm}^{0.1\%} = 0.83$ cm^{-1} (*Worthington Enzyme Manual*, 1977). Microsomal leucine aminopeptidase, obtained from Sigma Chemical Co., was used directly in kinetic experiments.

Enzyme Assays. Cytosolic leucine aminopeptidase was assayed at 25 °C in triethanolamine hydrochloride buffer (7.5×10^{-3} M, pH 8.4) containing $MgCl_2$ (5×10^{-3} M). Hydrolysis of the substrate L-leucine *p*-nitroanilide, dissolved in 100 μL of dimethyl sulfoxide (Me_2SO), was determined by following the change in absorbance at 405 nm [$\Delta\epsilon_{405} = 9620$ M^{-1} cm^{-1} (Wachsmuth et al., 1966a,b)] after addition of enzyme (15.4 μg) to the assay mixture (3 mL final volume). Activity of microsomal leucine aminopeptidase was determined by using L-leucine *p*-nitroanilide as a substrate (dissolved in 100 μL of Me_2SO) at 25 °C in 0.05 M potassium phosphate buffer, pH 7.2. The reaction was started by addition of enzyme (9.8 μg) to the test solution (3 mL final volume).

0006-2960/82/0421-4177$01.25/0

Molecular weights of 255000 (Melius et al., 1970) and 280000 (Auricchio & Bruni, 1964) were used to estimate molar concentrations of the cytosolic and microsomal enzymes, respectively.

Synthesis of L-*Leucinal Semicarbazone.* L-Leucinal semicarbazone was prepared by enzymatic oxidation of L-leucinol (2.5 mmol) by horse liver alcohol dehydrogenase, trapping the enzymatically formed aldehyde with semicarbazide and recycling the oxidized coenzyme chemically with FMN (L. Andersson and R. Wolfenden, unpublished results). L-[1-^{2}H]Leucinol for the synthesis of deuterium labeled L-leucinal semicarbazone was prepared from L-leucine methyl ester (11.3 mmol) by reduction with a 5-fold molar excess of sodium tetradeuterioborate: yield 4.2 mmol (37%); R_f 0.84 [on cellulose in 1-butanol/acetic acid/water (50:20:30 v/v)]; NMR (in D_2O) δ 0.9 [6 H, q, J(vicinal) = 6 Hz, J < 2 Hz, CH-(CH_3)$_2$], 1.2 (2 H, m, CH_2CH<), 1.7 (1 H, m, CH<), and 2.9 (1 H, t, J = 8 Hz, CH_2CHCD_2). L-[^{2}H]Leucinal semicarbazone was prepared as follows. L-Leucinol (1 mmol), NAD^+ (0.4 mmol), FMN (4 mmol), semicarbazide (60 mmol), and horse liver alcohol dehydrogenase (100 mg) were added to a solution (200 mL) containing 75 mM sodium pyrophosphate (final pH 8.7). After reduction for 70 h in darkness at room temperature additional amounts of FMN (2 mmol), NAD^+ (0.2 mmol), and alcohol dehydrogenase (100 mg) were added to the mixture, and the pH was adjusted to 8.7. The reaction was complete after about 120 h and the product was isolated as described by L. Andersson and R. Wolfenden (unpublished results): yield 0.20 mmol (20%); R_f 0.82 [on cellulose in 1-butanol/acetic acid/water (50:20:30 v/v)]; NMR (in D_2O) δ 0.8 [6 H, d, J = 6 Hz, CH(CH_3)$_2$], 1.3 (3 H, m, CH_2CH<), and 4.0 (1 H, t, J = 7 Hz, CH_2CHCD=).

Synthesis of L-*Leucinal.* To L-leucinal semicarbazone (0.128 mmol), dissolved in 2.6 mL of 38% (v/v) aqueous methanol containing 0.1 M HCl, was added a 10-fold molar excess of formaldehyde. After hydrolysis for 3 h at room temperature, the solution was taken to dryness (30 °C) and redissolved in water (20 mL). The solution was adjusted to pH 3 with dilute KOH and was then extracted several times with chloroform (total volume 225 mL) to remove excess formaldehyde and formaldehyde semicarbazone formed during hydrolysis. The aqueous phase was evaporated to dryness (30 °C), and the residue was redissolved in water. Evaporation with solvent water was repeated several times to remove last traces of formaldehyde. The residue was finally dried over P_2O_5 under vacuum: yield 0.062 mmol (48%); R_f 0.93 [on cellulose in 1-butanol/acetic acid/water (50:20:30 v/v)]; NMR (in D_2O) δ 0.9 [6 H, q, J(vicinal) = 6 Hz, J < 2 Hz, CH-(CH_3)$_2$], 1.5 (3 H, m, CH_2CH<), 3.2 (1 H, m, >$CHNH_2$), 5.1 [1 H, d, J = 5 Hz, CH(OH)$_2$], and 9.6 (1 H, s, CHO). The signal of the aldehyde proton weak in water or in D_2O was observed by using a 250-MHz Bruker NMR spectrometer. It is worth noting that the free aldehyde did not migrate on silica-coated thin-layer chromatography sheets developed in various mixtures of chloroform and methanol; it was also difficult to visualize α-aminoaldehydes on silica gel by spraying with ninhydrin. The amino function of the aldehyde reacted normally with ninhydrin in solution and on cellulose plates. L-[1-^{2}H]Leucinal was prepared analogously from its semicarbazone (0.186 mmol): yield (by NMR, using pyrazine and dioxane as integration standards) 0.095 mmol (51%); NMR (in D_2O) δ 0.9 [6 H, q, J(vicinal) = 6 Hz, J < 2 Hz, CH-(CH_3)$_2$], 1.5 (3 H, m, CH_2CH<), and 3.2 (1 H, t, J = 8 Hz, CH_2CHCHO).

Table I: Equilibrium Constants for Hydration of α-Aminoaldehydes Determined by NMR Measurements

aldehydes	$K_{hydration}$
L-leucinal[a]	39.6
glycinal[a]	47.0
N^α-acetyl-L-phenylalaninal[b]	9 ± 1
benzamidoacetaldehyde[c]	11.82

[a] $K_{hydration}$ = [hydrate]/[aldehyde] was determined at room temperature in D_2O at pH 3–4, from the NMR integrals of the 1 hydrogen of the hydrate and the free aldehyde, using a Bruker 250-MHz NMR spectrometer, at 25 °C. [b] Chen et al. (1979). [c] Lewis & Wolfenden (1977a).

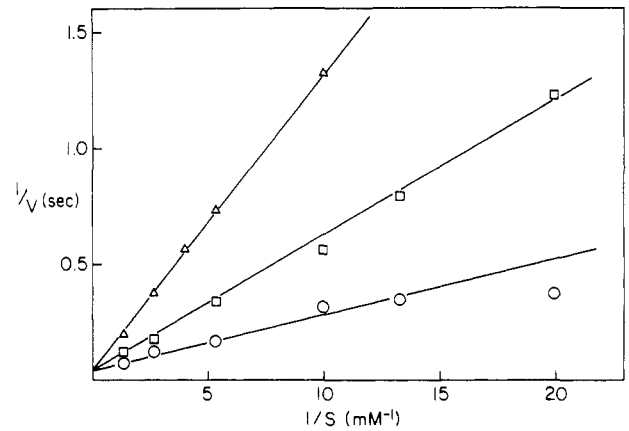

FIGURE 1: Double-reciprocal plot of the rate of hydrolysis of L-leucine *p*-nitroanilide by microsomal leucine aminopeptidase (1.2×10^{-8} M) as a function of substrate concentration in the absence (○) and in the presence of 9.8×10^{-7} M (□) and 4.9×10^{-6} M (△) L-leucinal. Rate measurements were carried out in potassium phosphate buffer (50 mM, pH 7.2) containing 3.4% (v/v) Me_2SO, at 25 °C.

N^α-Acetyl-L-leucinal was prepared by hydrolysis of N^α-acetyl-L-leucinal semicarbazone according to the method described for synthesis of N^α-acetyl-L-phenylalaninal (L. Andersson and R. Wolfenden, unpublished results).

Synthesis of Glycinal. Aminoacetaldehyde diethyl acetal, dissolved in water, was hydrolyzed after adjusting the pH of the solution to 1 with HCl (Westerik & Wolfenden, 1972). After complete hydrolysis, the solution was evaporated to dryness (30 °C): NMR (in D_2O) δ 2.9 (2 H, d, J = 6 Hz, CH_2HN_2), 5.2 [1 H, t, J = 6 Hz, $CH(OH)_2$], and 9.5 (s, CHO). The signal of the aldehyde proton was observed by using a 250-MHz Bruker NMR spectrometer.

Results

α-Aminoaldehydes, stored dry at −20 °C, were dissolved in water before use. L-Leucinal was found to be stable over a period of several days in dilute solution at pH 3 in the cold. Equilibrium constants for covalent hydration of leucinal and glycinal, observed by comparing integrated proton magnetic resonance spectra of the aldehyde and hydrate, were substantially larger than those observed previously for hippuraldehyde (Lewis & Wolfenden, 1977a) and *N*-acetylphenylalaninal (Chen et al., 1979), as shown in Table I. Only 2.5% of the total leucinal in dilute aqueous solution was found to be present as the free aldehyde.

When L-leucinal was tested as an inhibitor of porcine kidney microsomal leucine aminopeptidase, strong inhibition was observed as shown in Figure 1. The observed K_m for the substrate L-leucine *p*-nitroanilide at pH 7.2 and 25 °C was 5.2×10^{-4} M. K_i values observed for the amino acid, the amino alcohol, the aminoaldehyde semicarbazone, and the acetylated aldehyde were comparable in magnitude (Table II). In

Table II: Observed Dissociation Constants for Complexes with Microsomal Leucine Aminopeptidase at pH 7.2, 25 °C

inhibitor	K_i[a] (mM)
L-leucine *p*-nitroanilide (K_m)	0.52
L-leucinal	0.00076
L-leucinol	4.4
L-leucine[b]	2.95
L-leucinal semicarbazone	0.23
N^{α}-acetyl-L-leucinal	0.43

[a] In all cases, inhibition was shown to be competitive. L-Leucine *p*-nitroanilide (0.05–0.8 mM) was used as a substrate. For the uninhibited enzyme, k_{cat} and K_m values of 21.4 ± 2.9 s^{-1} and 0.52 ± 0.13 mM, respectively, were determined. Data were subjected to least-squares regression analyses. [b] At 37 °C, pH 7, from Wachsmuth et al. (1966a,b).

Table III: Observed Dissociation Constants for Complexes with Cytosolic Leucine Aminopeptidase at pH 8.4, 25 °C

inhibitor	K_i[a] (mM)
L-leucinol *p*-nitroanilide (K_m)	0.77
L-leucinal	0.00006
glycinal	0.68

[a] In both cases, inhibition was shown to be competitive. L-Leucine *p*-nitroanilide (0.16–5 mM) was used as a substrate. For the uninhibited enzyme k_{cat} and K_m values of 2.75 ± 0.09 s^{-1} and 0.77 ± 0.1 mM, respectively, were determined. Data were subjected to least-squares regression analyses.

contrast, the apparent K_i of the aminoaldehyde was several orders of magnitude lower (Table II). If the free aldehyde is the actual form of the inhibitor that combines with the enzyme, its true K_i value is 1.9×10^{-8} M, about 27 000-fold lower than the K_m value of the nitroanilide substrate.

For porcine kidney cytosolic leucine aminopeptidase, the apparent K_i value observed for L-leucinal was even lower (Table III). Corrected for hydration, the K_i value for the free aldehyde would be 1.5×10^{-9} M, lower than the K_m value of L-leucine *p*-nitroanilide by a factor of almost 10^6. Glycinal, in contrast, is a weak inhibitor (Table III).

Discussion

The unusual affinity of leucine aminopeptidases for leucinal suggests that inhibition is related to the specific mechanism of action of this enzyme. Transition state analogues are expected to reflect the catalytic specificiity of the enzymes they inhibit. Accordingly the very weak binding observed for glycinal seems understandable in view of the very slow turnover of glycine derivatives by leucine aminopeptidase (Smith & Spackman, 1955).

In considering the likely structure of the inhibitory complex, and the mechanism of substrate transformation to which this might correspond, it may be noted that the relative binding affinities observed for the various leucine derivatives (Table II) are comparable with those observed for various hippuric acid derivatives bound by papain (Westerik & Wolfenden, 1972). Inhibitory aldehydes, resembling specific substrates, are known to form hemiacetals at the active sites of papain (Lewis & Wolfenden, 1977b; Clark et al., 1977), chymotrypsin (Lowe & Nurse, 1977; Chen et al., 1979; Wyeth et al., 1980) and a bacterial protease (Brayer et al., 1979), as had been postulated when these inhibitors were originally designed (Westerik & Wolfenden, 1972; Thompson, 1973). By analogy, these observations strengthen the likelihood that leucinal is bound covalently by aminopeptidases and suggest that this enzyme also acts by a double-displacement mechanism. It remains to be established, however, that the active site of leucine aminopeptidase contains a nucleophilic residue appropriate for the addition reaction postulated above. There are conflicting reports concerning the enzyme's sensitivity to mercurials (Smith & Spackman, 1955; Bryce & Rabin, 1964), and the enzyme is not inactivated by diisopropyl fluorophosphate (Smith & Spackman, 1955).

Alternative possibilities are that leucinal is bound as the free aldehyde or as the covalent hydrate. The covalent hydrate of the aldehyde would be analogous in structure to a tetrahedral intermediate formed by direct water attack on normal substrates, and the observation of such a structure in the E–I complex would tend to support a mechanism of this kind. However, leucinol might be expected to share, at least in part, any special binding properties of the covalent hydrate. In fact, leucinol is bound no more tightly than the product leucine (Table II), so that no special binding affinity is apparent. If leucinal were bound instead as the intact aldehyde (not as an adduct involving water or an active-site nucleophile), then its binding might be expected to reflect, almost in full, the 1.37-fold difference in equilibria of hydration between the unlabeled aldehyde and [1-^{2}H]aldehyde (Lewis & Wolfenden, 1977a). Because of the greater difficulty of stripping covalently bound water from the deuterioaldehyde (Lewis & Wolfenden, 1977b), the protioaldehyde would appear to be substantially more tightly bound at equilibrium. Preliminary experiments suggest that unlabeled leucinal and [1-^{2}H]leucinal are very similar in the inhibition that they produce. Accordingly it seems probable that the aldehyde is bound as a covalent adduct, as postulated in its design.

Expressed as an equilibrium constant involving the free aldehyde, the affinity of L-leucinal for leucine aminopeptidase exceeds that of the substrate leucine *p*-nitroanilide, by factors of 270 000 and 508 000 for the microsomal and cytosolic enzymes, respectively. As expected for an inhibitor whose effectiveness is based on specific binding interactions that are manifested in the transition state, the affinity of the cytosolic enzyme for leucinal is about 4 orders of magnitude greater than for glycinal, reflecting the strong catalytic preference of this enzyme for substrates that contain leucine rather than glycine. Aminoaldehydes seem likely to prove useful as specific eluents in the analytical separation of aminopeptidases by transition state affinity jump chromatography (Andersson & Wolfenden, 1980) and as tools for investigating the physiological function and mechanism of action of aminopeptidases in excitable tissue.

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Halibut Muscle 3-Phosphoglycerate Kinase. Chemical and Physical Properties of the Enzyme and Its Substrate Complexes†

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ABSTRACT: An efficient procedure for the purification of 3-phosphoglycerate kinase (PGK) from Pacific halibut muscle is described. The molecular weight (43 500) and specific activity are similar to those of other species of PGK. The isoelectric point (>9.5) is more than 1.4 pH units higher than that reported for mammalian muscle PGK. The reaction of the seven thiol groups with 5,5′-dithiobis(2-nitrobenzoic acid) (Nbs_2) is kinetically biphasic; reaction at a single fast-reacting thiol inactivates the enzyme. The binding of all substrates and products to PGK was observed by ^{31}P NMR. 1,3-Diphosphoglycerate (1,3-P_2G) is more tightly bound than is any of the other reaction components. Unlike 1,3-P_2G in aqueous solution, the complex with PGK is protected from hydrolysis over a period of weeks. The ^{31}P chemical shifts of this complex are insensitive to pH which suggests that solvent water is excluded from the substrate-bound cleft. As with yeast PGK, the equilibrium constant for the phosphoryl transfer reaction is near unity in the enzyme site environment in contrast to a value of approximately 10^3 (in favor of ATP) in aqueous solution. Since the ternary complex equilibrium ^{31}P NMR spectrum can be accounted for entirely on the basis of the various binary complex spectra, there is no compelling evidence for the involvement of a stoichiometrically substantial phosphoenzyme intermediate.

3-Phosphoglycerate kinase (ATP:3-phospho-D-glycerate 1-phosphotransferase, EC 2.7.2.3) catalyzes the reversible, magnesium ion dependent phosphoryl transfer from 1,3-diphosphoglycerate (1,3-P_2G)[1] to ADP (eq 1) in an important

$$1,3\text{-}P_2G + ADP \overset{Mg^{2+}}{\rightleftharpoons} 3\text{-}PG + ATP \qquad (1)$$

ATP generation step of the glycolytic pathway. PGK has been purified from several sources (Krietsch & Bücher, 1970; Yoshida & Watanabe, 1972; Okonkwo et al., 1973; Scopes, 1975a,b; Johnson et al., 1976b; De & Kirtley, 1977). Insight into the structure and function relationships operative in PGK catalysis has been advanced by X-ray crystallographic studies of the enzymes from yeast (Bryant et al., 1974) and from horse muscle (Blake & Evans, 1974), by recent mechanistic studies (Johnson et al., 1976a; Wrobel & Stinson, 1978; Scopes, 1978a,b; Webb & Trentham, 1980) of both yeast and muscle PGK, and by the total sequencing of horse muscle PGK (Banks et al., 1979).

This laboratory has previously studied the detailed catalytic properties of several glyceraldehyde-3-phosphate dehydrogenase (GPDH) enzymes with most recent emphasis on the enzymes from sturgeon and halibut muscle (Seydoux et al., 1973; Seydoux & Bernhard, 1974; Schwendimann et al., 1976; Bernhard et al., 1977; Malhotra & Bernhard, 1981). The sarcoplasmic fluid of white fish muscle is an abundant source of the glycolytic enzymes and is nearly devoid of other protein components. For reasons discussed elsewhere (Seydoux et al., 1973), the fish muscle glycolytic enzymes are particularly useful for investigations of the regulatory processes which modulate the catalytic function.

The molecular events which occur during the sequence of reactions by which glyceraldehyde 3-phosphate, inorganic

† From the Institute of Molecular Biology and Department of Chemistry, University of Oregon, Eugene, Oregon 97403. *Received January 8, 1982.* This investigation was supported by grants from the U.S. Public Health Service (2R01 GM 10451-13) and from the National Science Foundation (BMS 75-23297). K.R.H. was the recipient of a traineeship from the U.S. Public Health Service (5T01 GM 00715). This work has been submitted (by K.R.H.) in partial fulfillment of the requirements for the Ph.D. degree at the University of Oregon.

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[1] Abbreviations: 1,3-P_2G, 1,3-diphospho-D-glycerate; 3-PG, 3-phospho-D-glycerate; NAD^+ and NADH, nicotinamide adenine dinucleotide, oxidized and reduced forms, respectively; PGK, 3-phosphoglycerate kinase; GPDH, glyceraldehyde-3-phosphate dehydrogenase; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid, disodium salt; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; CM, carboxymethyl; $NaDodSO_4$, sodium dodecyl sulfate; Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid); Nbs, 2-nitro-5-thiobenzoate.

0006-2960/82/0421-4180$01.25/0