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4-HYDROXY-3-NITROPHENYLGLYOXAL

A CHROMOPHORIC REAGENT FOR ARGINYL RESIDUES IN PROTEINS

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Summary

The chromophoric reagent, 4-hydroxy-3-nitrophenylglyoxal, is highly selective for the modification of arginine in aqueous solution at pH 7—9. The reagent also inactivates creatine kinase (ATP:creatine *N*-phosphotransferase, EC 2.7.3.2) in a manner analogous to that reported with phenylglyoxal.

Arginyl residues are now widely recognized to play a general role in the binding of anionic substrates and cofactors to enzyme active sites [1]. Phenylglyoxal is one of the arginine-specific reagents which has made this conclusion possible [2]. There appears to be a high degree of selectivity with phenylglyoxal, for many enzymes can be inactivated by modification of one arginyl residue per enzyme protomer or active site [3—8]. We have attempted to take advantage of this selectivity by synthesizing 4-hydroxy-3-nitrophenylglyoxal, a chromophoric analog of phenylglyoxal. This reagent demonstrates the same selectivity as phenylglyoxal, and the adducts it forms with arginine are reasonably stable. Thus, these properties should facilitate peptide isolation and identification and make easier the determination of primary sequences involving essential arginyl residues. The reagent could also be used to probe the environment of essential arginines [9], and as a probe of protein-small molecule interactions [10]. A preliminary report of this work has appeared [11].

4-Hydroxy-3-nitrophenylglyoxal was synthesized by the procedure of Fodor and Kovács [12] with some modifications: 0.1 mol 4-hydroxy-3-nitroacetophenone [13] was refluxed with 0.1 mol H_2SeO_3 in 100 ml dioxane for 4 h. After filtering while hot and removal of the solvent by rotary-evaporation, the resulting orange-brown oil was refluxed for 3 h in 275 ml 25% aqueous

ethanol, concentrated to half its original volume, filtered while hot, and finally concentrated to 50 ml. On cooling, 15.9 g 4-hydroxy-3-nitrophenylglyoxal (75% yield) was isolated. The reagent recrystallizes from hot water as the monohydrate, m.p. 97–98.5°C. Analysis: Found (PCR, Inc., Gainesville, FA, U.S.A.): C, 45.11%; H, 3.36%; N, 6.39%. Calcd. for $C_8H_5NO_5 \cdot H_2O$: C, 45.08%; H, 3.31%; N, 6.57%. At pH 8, 4-hydroxy-3-nitrophenylglyoxal absorbs maximally at 395 nm and 316 nm, with molar absorption coefficients of $4.75 \cdot 10^3$ $M^{-1} \cdot cm^{-1}$ and $1.88 \cdot 10^4$ M^{-1} cm^{-1}, respectively, while at pH 3 it absorbs maximally at 338 nm with a molar absorption coefficient of $2.60 \cdot 10^3$ $M^{-1} \cdot cm^{-1}$. The phenolic proton has a pK_a of approx. 4.8 at 25°C.

4-Hydroxy-3-nitrophenylglyoxal was reacted with those amino acids present in a standard amino acid calibration mixture at various pH values (Table I). At each pH, arginine was modified most rapidly. The rate of modification of arginine was quite pH-dependent, for the same extent of modification was observed after 24 h at pH 7.0, but only 1 h at pH 8.0, and 15 min at pH 9.0. The selectivity for arginine was greatest at pH 9.0, when, after only 15 min, arginine was 73% modified and all other amino acids were less than 7% reacted. In separate experiments, when the reagent was reacted with asparagine, glutamine and tryptophan for 1 h at pH 8.0, no loss of these amino acids was detected.

The modification of arginine by 4-hydroxy-3-nitrophenylglyoxal was followed by ion-exchange chromatography (Fig. 1). The loss of arginine with time coincided with the formation of two ninhydrin-positive peaks, the first (peak I, Fig. 1) eluting immediately after the void volume and the second (peak II, Fig. 1) before lysine. The color calibration constants for both peaks are apparently different than that for arginine, for the sum of the areas of peaks I and II was significantly less than the decrease in the area under the arginine peak at any given time of modification. The adducts between arginine

TABLE I

MODIFICATION OF STANDARD AMINO ACIDS BY 4-HYDROXY-3-NITROPHENYLGLYOXAL

Solutions containing all the above amino acids (0.25 mM each) were modified by 15 mM 4-hydroxy-3-nitrophenylglyoxal at 25°C at the indicated pH. Buffers were 0.45 M *N*-(2-acetamido)iminodiacetic acid at pH 7.0, and 0.45 M *N,N*-bis(2-hydroxyethyl)glycine at pH 8.0 and 9.0. After reaction for the indicated time, aliquots were quenched by dilution into 0.2 M citrate (pH 2.2) and analyzed on a Beckman 120C amino acid analyzer. Values are reported as a percent of a control subjected to identical conditions but in the absence of modifying reagent.

Amino acid	pH 7.0, 24 h	pH 8.0, 1 h	pH 9.0, 15 min
Asp	102	99	103
Thr	102	92	95
Ser	102	93	98
Glu	102	96	98
Pro	100	99	97
Gly	100	89	97
Ala	102	95	101
Cys/2	92	77	93
Val	99	95	99
Met	99	85	96
Ile	102	98	99
Leu	100	95	99
Tyr	98	94	94
Phe	101	96	96
Lys	99	90	93
His	74	80	93
Arg	31	34	27

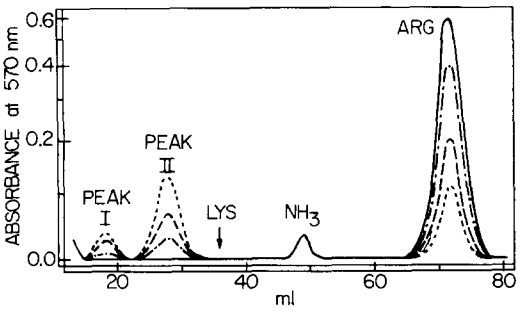

Fig. 1. Chromatography of 4-hydroxy-3-nitrophenylglyoxal/arginine reaction mixtures. L-Arginine, at 2.5 mM, was reacted with 30 mM 4-hydroxy-3-nitrophenylglyoxal in 0.5 M *N,N*-bis(2-hydroxyethyl)-glycine (pH 8.0), at 25° C. At the indicated times, aliquots were quenched by dilution into 0.2 M citrate (pH 2.2) and run on the short column (0.9 × 7.5 cm) of a Beckman 120C amino acid analyzer. Before chromatography the samples were extracted with ether to remove excess reagent, which otherwise elutes at the void volume of the column and interferes with the baseline. Shown are samples which were reacted with 4-hydroxy-3-nitrophenylglyoxal for 10 (— · — ·), 30 (— — —) and 60 (- - - -) min before analysis. Also shown is a control subjected to the same conditions but in the absence of reagent (——). The arrow indicates the normal elution position of lysine.

and 4-hydroxy-3-nitrophenylglyoxal were unstable to hydrolysis with 6 M HCl. When a sample containing reagent-modified arginine (1 μmol arginine originally, 88% modified) was maintained in 6 M HCl at 110°C for 24 h, the amount of arginine increased to 65% of its original value. In addition, peaks I and II disappeared and a new peak appeared upon amino acid analysis which chromatographed at a position identical to that of ornithine. This suggests that the adduct(s) decomposed on acid hydrolysis by two different routes, one to regenerate arginine and a second to give ornithine.

We have observed that 4-hydroxy-3-nitrophenylglyoxal undergoes a spontaneous acid-releasing process when maintained at pH 8 or above. This side-reaction does not pose a problem if freshly prepared solutions of reagent are used, because when modification of 1 mM arginine by 15 mM 4-hydroxy-3-nitrophenylglyoxal at pH 8.0 was followed by amino acid analysis, the loss of arginine was pseudo first-order for more than two half-lives.

The spectra of 4-hydroxy-3-nitrophenylglyoxal as a function of pH are straightforward, and a simple acid-base equilibrium is obvious from pH-absorbance titration data at either 395 nm or 316 nm. However, the spectral properties were altered when the reagent was reacted with arginine. For example, when 0.1 mM reagent was maintained for 4 h in 50 mM *N*-acetyl-L-arginine (pH 9.0) before spectral titration, the longer wavelength, λ_{max}, shifted to 405 nm. The molar absorption coefficient of this longer wavelength band was essentially the same in the 4-hydroxy-3-nitrophenylglyoxal:*N*-acetyl-L-arginine adduct(s) as in the free reagent, but that of the spectral band in the 316 nm region decreased to $1.09 \cdot 10^4\ M^{-1} \cdot cm^{-1}$. The pH-absorbance titration of either band indicates that more than one acid-base equilibria are involved. We are currently attempting to study the spectral properties of the two products of the reaction of 4-hydroxy-3-nitrophenylglyoxal with arginine (Fig. 1) in an effort to resolve these problems.

Creatine kinase has one essential arginyl residue per subunit which is selectively modified by phenylglyoxal [3]. 4-Hydroxy-3-nitrophenylglyoxal also rapidly inactivates creatine kinase, for when the enzyme was reacted at

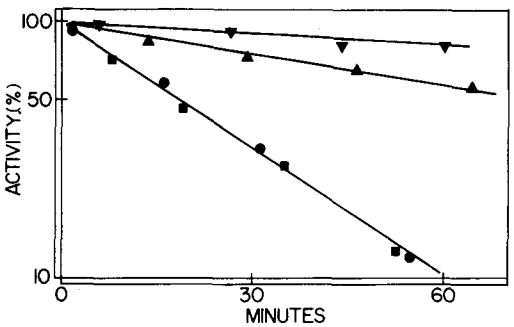

Fig. 2. Changes in the activity of creatine kinase in 50 mM *N,N*-bis(2-hydroxyethyl)glycine (pH 8.8), at 25°C, vs. time on modification with 1.0 mM 4-hydroxy-3-nitrophenylglyoxal. The enzyme was modified either in the absence of substrates (●), or in the presence of 40 mM phosphocreatine (■), 20 mM ATP (▲), or 20 mM Mg·ATP (▼). The control retains full activity over this period of time.

25°C with 1 mM reagent at pH 8.8, activity was lost by a first-order process ($t_{1/2}$ = 19 min) (Fig. 2). When several other concentrations of reagent were used, inactivation was pseudo first-order in 4-hydroxy-3-nitrophenylglyoxal with a second-order rate constant of approx. 35 $M^{-1} \cdot min^{-1}$. The effects of the presence of substrates on the inactivation of creatine kinase by 4-hydroxy-3-nitrophenylglyoxal were very similar to results obtained with phenylglyoxal [3], i.e., phosphocreatine offered no protection, while ATP afforded significant protection and Mg·ATP provided almost complete protection (Fig. 2).

The loss of 85% of the activity of creatine kinase coincided with the incorporation of 1.1 4-hydroxy-3-nitrophenylglyoxals per subunit, if the tenuous assumption is made that the molar absorption coefficient of the reagent in the 395 nm region is similar in the free and protein-bound forms (vide infra). From the results of the modification of free amino acids, it is most likely that the incorporation of 4-hydroxy-3-nitrophenylglyoxal into creatine kinase involves the modification of the essential arginyl residue per subunit. This could not be corroborated by amino acid analysis after hydrolysis with 6 M HCl, however, since very little loss of arginine was detected when this experiment was carried out. This is consistent with the studies on reagent-modified arginine mentioned above. The further characterization of the inactivation of creatine kinase by 4-hydroxy-3-nitrophenylglyoxal is currently under investigation.

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References

1 Riordan, J.F., McElvany, K.D. and Borders, C.L., Jr. (1977) Science 195, 884–886
2 Takahashi, K. (1968) J. Biol. Chem. 243, 6171–6179
3 Borders, C.L., Jr. and Riordan, J.F. (1975) Biochemistry 14, 4699–4704
4 Lobb, R.R., Stokes, A.M., Hill, H.A.O. and Riordan, J.F. (1976) Eur. J. Biochem. 70, 517–522
5 Kantrowitz, E.R. and Lipscomb, W.N. (1976) J. Biol. Chem. 251, 2688–2695
6 Salvo, R.A., Serio, G.F., Evans, J.E. and Kimball, A.P. (1976) Biochemistry 15, 493–497
7 Armstrong, V.W., Sternbach, H. and Eckstein, F. (1976) FEBS Lett. 70, 48–50

8 Berghäuser, J. (1975) Biochim. Biophys. Acta 397, 370—376
9 Horton, H.R. and Koshland, D.E., Jr. (1965) J. Am. Chem. Soc. 87, 1126—1132
10 Vallee, B.L., Riordan, J.F., Johansen, J.T. and Livingston, D.M. (1971) Cold Spring Harbor Symp. Quant. Biol. 36, 517—531
11 Borders, C.L., Jr., Pearson, L.J., McLaughlin, A.E., Vasiloff, J., Gustafson, M.E. and Morgan, D.J. (1978) Fed. Proc. 37, 1510
12 Fodor, G. and Kovács, Ö. (1949) J. Am. Chem. Soc. 71, 1045—1048
13 Pope, G. (1912) Proc. Chem. Soc. 28, 331—332